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# Superovulation And Infertility In Immature Rats

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**LA THÈSE A ÉTÉ  
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**SUPEROVULATION AND INFERTILITY IN IMMATURE RATS**

by

**Elizabeth Anne Walton**

**Department of Physiology**

**Submitted in partial fulfillment  
of the requirements for the degree of**

**Doctor of Philosophy**

**Faculty of Graduate Studies  
The University of Western Ontario**

**London, Ontario**

**March 1983**

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Inai gaukara lauegu sinana bona tamana bona lauegu  
adavana lau ura henia.\*

\* To my parents and husband - translated from Hiri Motu.

## ABSTRACT

The purpose of this study was to examine the possible causes of infertility in immature rats following superovulation (SOV) with pregnant mares' serum gonadotrophin (PMSG). Defects could occur in one or more of the processes from oocyte maturation through ovulation, fertilization and implantation to foetal development, or the fault could be in the oviductal or uterine environment, or in any combination of these. Experiments were designed to identify the mechanism of this infertility and the areas looked at included the timing of ovulation, oocyte normality, fertilization, oviductal transport, early embryo development and implantation.

In the initial experiments SOV with either a single injection of PMSG or continuous infusion of follicle-stimulating hormone (FSH) was compared, and in some rats the period of action of PMSG was restricted to 58 h by use of a specific antiserum (a/s). Pregnancy was observed in most FSH-infused rats, whereas following SOV with PMSG pregnancy failed prior to day 5 (as judged by the absence of blastocysts in the uteri). Serum and ovarian oestradiol concentrations were significantly elevated in SOV rats and the results suggested that the infertility associated with PMSG was produced, at least in part, via increased ovarian oestradiol secretion. Small increases in embryo recovery rates were observed when postovulatory oestradiol concentrations were reduced by inhibitors of steroid synthesis. Use of the PMSG a/s was unable to correct all the abnormalities caused by the PMSG. However, pregnancy did continue to term in some animals; the

major loss of pregnancy in the remaining rats occurred around the time of implantation.

Various stages of preimplantation development were studied to determine the contribution each might make to the infertility following superovulation. It was found that ovulation in superovulated rats occurred over a long time span from 22 h after PMSG onwards, with a burst of ovulations at 64-70 h; the latter time was comparable to that seen in control rats. In vivo fertilization rates of oocytes in cumulus from superovulated rats were lower, probably due to the long time span of ovulation. However, the developmental capacity of oocytes collected from the oviduct of superovulated rats shortly after ovulation was equal to that of oocytes from control rats, when assessed by transfer to a synchronized recipient.

In superovulated rats the majority of embryos was lost between days 1 and 3 of pregnancy. Administration of a/s partially overcame this loss, but retention of the oocytes within the oviduct by means of ligatures was unable to prevent the loss and actually increased the proportion of degenerate embryos. Use of PMSG a/s ensured the presence of blastocysts in the uterus on day 5 of pregnancy, but implantation only occurred in ~ 50% of rats. The ability of the uteri to undergo decidualization was frequently impaired, but transfer studies showed that the blastocysts were normal.

The results of this research suggest that the infertility seen after SOV results primarily from changes in the maternal environment, resulting from excessive gonadotrophic stimulation. Excessive oestrogen secretion was particularly implicated. The preovulatory elevation in oestrogen concentrations appeared to prevent implantation

in many rats and elevated postovulatory oestrogen concentrations caused loss of embryos by accelerated transport. Oocytes and blastocysts from superovulated rats are normal if transferred to suitable recipients.

## ACKNOWLEDGEMENTS

No ship can be sailed single-handed although the captain may hold ultimate responsibility. Similarly with this thesis, although the author may be held responsible for its contents, its voyage would have ended at the bottom of the ocean had it not been for the help of many people. In particular I would like to thank the chief navigator, Dr. David Armstrong, whose knowledge of the oceans was invaluable and who was not deterred by the occasional seasickness of the author. My thanks must also go to Dr. Bevan Miller whose work initiated the voyage and to other pilots who assisted with navigation. In particular I must mention Drs. Tom Kennedy and Gareth Evans, the former's "Pommie bashing" never prevented him from giving much valuable advice and the latter's interest in reproductive glands above the waist was frequently educational. Drs. Kline, Mogenson and Weick also advised ably when called upon to do so.

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I would also like to thank Dr. Vickery of Syntex, Palo Alto, CA for his generous advice concerning the synchronization of adult rats

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The work on fertilization (Chapter 7) was done in collaboration with Dr. Gareth Evans and the decidualization studies (Chapter 8) were done in collaboration with Dr. Tom Kennedy.

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# GLOSSARY

4AA	4-acetoxy-4-androstene-3,17-dione
AGP	aminoglutethimide phosphate
a/s	antiserum
CL	corpora lutea
DBS	Dulbecco's buffered phosphate saline
E <sub>2</sub>	oestradiol
FSH	follicle-stimulating hormone
g	gram
h	hour
hCG	human chorionic gonadotrophin
hMG	human menopausal gonadotrophin
i.m.	intramuscular
i.p.	intraperitoneal
i.u.	international units
i.v.	intravenous
L-E	Long-Evans
LH	lutēinizing hormone
LHRH	lutēinizing hormone-releasing hormone
μl	microlitre
ml	millilitre
ng	nanogram
NGS	normal goat serum
oestradiol	oestradiol-17β

PBS	phosphate buffered saline
P <sub>4</sub>	progesterone
pg	picogram
PMSG	pregnant mares' serum gonadotrophin
RIA	radioimmunoassay
saline	0.9% sodium chloride
s.c.	subcutaneous
S-D	Sprague-Dawley
S.E.M.	standard error of the mean
SOV	superovulation/superovulated
wt.	weight
~	approximately
>	greater than
<	less than

## CHAPTER 1

### INTRODUCTION

Superovulation may be defined as a marked increase in the number of follicles ovulating at any given time in an animal's life. This may include treatments that induce many more ovulations than occur in normal cycling animals or the stimulation of ovulation in prepubertal and anoestrous animals. The technique of superovulation and its study may have various benefits, including an increase in the understanding of the basic function of the ovary, particularly the pattern of maturation of follicular oocytes and its hormonal control (Phillippo, 1968). It enables one of the factors affecting pregnancy to be varied and the number of offspring to be increased. In domestic livestock this serves to multiply genetically superior stock and allows for progeny testing and a decreased generation interval (Gordon, 1975).

Superovulation was first described more than 50 years ago (P.E. Smith, 1926; Engle, 1927; Smith and Engle, 1927) when it was reported that daily intramuscular implants of anterior pituitary tissue to the rat or mouse resulted in the ovulation of a number of follicles well in excess of that observed at spontaneous ovulation. Since that time a great number of studies have been conducted in an effort to determine both the optimal conditions for superovulation

and the mode of action of gonadotrophic preparations.

Most regimens for the induction of superovulation involve the use of gonadotrophins prepared from pituitaries (FSH and LH), urine (hMG) or serum (PMSG), although there are reports in the literature of other methods, including X-irradiation of the ovaries (Hahn and Morales, 1964; Mandl, 1964). In addition it has been suggested that modifying steroid feedback could provide improved gonadal function and increased ovulation rates, in a more controlled manner than the present use of exogenous gonadotrophins (Land, 1979).

Fertility following superovulation has frequently been questioned but it has not been systematically studied. Decreased fertility or infertility could result from defects in oocyte maturation, ovulation, fertilization, implantation or foetal development; or the fault could be in the oviductal or uterine environment or in any combination of these.

The purpose of the present research was to study a rat model for infertility following superovulation, in order to appreciate the problems that may be encountered when large domestic animals and humans are superovulated. Due to difficulties in superovulating adult rats the immature rat was used, since it has been shown to be a good model for ovulation and pregnancy in the adult rat (Nutti et al, 1975). There is a great potential for superovulation followed by embryo transfer in domestic livestock, particularly cows, sheep and goats which usually produce only one or two offspring per breeding season. In humans induction of ovulation in anovulatory women and mild superovulation to increase the number of oocytes available for in vitro fertilization may be valuable methods for treating infertility. Therefore, although this research was performed in rats, the literature pertaining to problems

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associated with superovulation of large domestic livestock (cows, sheep and goats) and humans will be reviewed; data from other species will also be included when relevant.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Gonadotrophic Preparations

PMSG is widely used to induce superovulation in small laboratory animals (e.g., rats - Cole and Hart, 1930; rabbits - Pincus, 1940; hamsters - Greenwald, 1962) and in domestic livestock (reviewed by Betteridge, 1977; Betteridge and Moore, 1977), whereas hMG (Pergonal) is most widely used for induction of ovulation in humans (Steptoe and Edwards, 1970). Details of the different regimens used will not be described in detail as these are adequately covered in other reviews (e.g., Betteridge, 1977; Edwards and Steptoe, 1975; Soupart, 1981).

##### 2.1.1 Pregnant Mares' Serum Gonadotrophin

One of the most widely used gonadotrophins is the serum of pregnant mares (PMSG). Cole and Hart (1930) first observed that the serum of mares at various stages of pregnancy could produce superovulatory effects in immature rats very similar to those observed by Smith and Engle (1927). The properties and potency of this preparation were further studied (Cole *et al.*, 1932; Cole, 1936, 1937), various bioassays were suggested (Cole *et al.*, 1932; Cole and Saunders, 1935) and it was soon found that PMSG had an unusually long

circulating half-life in both mares and rabbits (Catchpole et al., 1935). That most of the effects of PMSG are directly attributable to a direct action and not indirectly to the actions of endogenous gonadotrophins was established by Cole et al. (1940), who showed that PMSG induced a response in hypophysectomized rats similar to that observed in the intact animal.

Cole and Goss (1943) were the first to suggest that PMSG was secreted by the specialized structures known as the endometrial cups, but it has only been recently established that PMSG is actually secreted by specialized chorionic girdle cells of trophoblastic origin which invade the endometrium 36-40 days after ovulation in the mare (Allen and Moor, 1972; Allen et al., 1973). For this reason it has been proposed that the hormone be renamed equine chorionic gonadotrophin (eCG) (Farmer and Papkoff, 1979).

Cole and co-workers first suggested that the dual gonadotrophic activity of PMSG depended on a single substance since they were unable to separate the activities by fractional precipitation (Cole et al., 1940; Goss and Cole, 1940). Later authors also found that the FSH and LH components of the hormone could not be separated (Hamburger, 1957) and in various biological tests PMSG was found to act like both LH and FSH (Simpson, 1961; Gospodarowicz and Papkoff, 1967), but with a greater LH-like activity (Licht et al., 1979). However, little was known until recently of the biochemistry of PMSG, particularly whether one or two gonadotrophins were responsible for its activity. The biochemical characterization of PMSG has been reviewed (Papkoff, 1978, 1981) and it has been suggested

that some of the molecular forms of the hormone have varying LH:FSH activity depending on the source (endometrial cups, trophoblastic cell culture medium or serum) (Aggarwal et al., 1980) or on the stage of gestation (González-Menció et al., 1978). It is now known that PMSG is a single molecule (Legault-Demaré et al., 1961; Gospodarowicz, 1972) with both  $\alpha$  and  $\beta$  subunits (Papkoff, 1974); the  $\beta$  subunit is responsible for both the LH- and FSH-like activities (Papkoff, 1974; Papkoff et al., 1978).

The carbohydrate content of PMSG is approximately 45%, of which 10-14% is sialic acid (Schams and Papkoff, 1972; Christokas and Bahl, 1979) and it has been suggested that the high sialic acid content protects the molecule from degradation in vivo. In vitro the data are conflicting with some authors claiming desialylation resulting in decreased potency (Farmer and Papkoff, 1978) and other authors claiming enhanced potency (Moyle et al., 1978; Moore and Ward, 1980). It has been suggested that variations in the sialic acid content may be reflected in the differing LH:FSH activities, but recent work (Moore and Ward, 1980) has disputed this since in vitro desialylation affected both LH and FSH activities to the same extent. The FSH activity of PMSG varies considerably depending on the species in which it is being tested and it has been suggested that this may be a function of the structural characteristics of the FSH receptor in different species (Moore and Ward, 1980).

The distribution and metabolism of injected PMSG has been studied to varying degrees in different species. Its half-life has been found to be approximately 21 h in sheep (McIntosh et al., 1975)



with no evidence that the ovary is required for its removal from the circulation. In cattle its rate of disappearance has two components, a faster one with a half-life of 45-50 h and a second slower one with a half-life of approximately 120 h (Schams et al., 1978; Menzer and Schams, 1979). Low doses injected directly into the ovary appear to bind to receptors there and produce a local effect (Betteridge, 1974). In mares following hysterectomy endogenous PMSG has a half-life of about 6 days (Cole et al., 1967) which is similar to that of injected PMSG in horses (Catchpole et al., 1935). In both rabbits and rats injected PMSG is said to have a half-life of approximately 26 h (Catchpole et al., 1936; Parlow and Ward, 1961), but recently such a long half-life has been questioned by Aggarwal and Papkoff (1981) who found that in male rats the disappearance curve had 2 exponents with half-lives of 0.2 and 6 h respectively. These authors suggested that the discrepancy reflected the greater purity of their preparation; alternatively there may be a difference in clearance rate between sexes. The relevance of the half-life is questionable since a considerable discrepancy has been shown between the half-life and biological life. Thus in the immature mouse Sasamoto (1962) has estimated the half-life to be 6 h whereas the biological life has been calculated to be 54-60 h (Sasamoto et al., 1972) or 6 days (Ladman, 1964); both authors having assessed this by the ability of PMSG to maintain follicles in a state in which hCG can induce ovulation. This discrepancy may reflect activity of tissue bound PMSG compared with that in the circulation. In addition to between species variation McIntosh et al. (1975) have suggested that the half-life of different preparations may vary according to the extent to which sialic acid was removed from the molecule during purification. It has been shown that desialylation

of PMSG reduced 400-fold its ability to induce ovulation in the hamster (Yang and Papkoff, 1973). In addition to affecting the half-life, differences between preparations have also been suggested as an explanation for varying responses, although Uberoi and Meyer (1966) have shown little variation in the response to different batches. Gordon (1975) has extensively reviewed the early literature on the widespread variations in response to PMSG in cattle. Unpublished evidence suggests that varying FSH:LH ratios are (Newcomb and Rowson, 1976) or are not involved (Stewart et al., 1976).

Systemic PMSG injections initially exert a luteotrophic effect in cattle (Henricks et al., 1973; Hallford et al., 1975), which has been attributed to its LH-like activity (Newcomb and Rowson, 1976). It is this LH-like activity that is probably responsible for the occasional premature ovulation of large follicles present at the time of PMSG injection (Newcomb and Rowson, 1976). PMSG does not appear to influence endogenous LH secretion directly (Hallford et al., 1975).

It has been suggested that PMSG could cause superovulation by recruiting more follicles into the growing population, by stimulating the development of follicles which are about to become atretic or by reversing the process of atresia (Dott et al., 1979). Dott et al. (1979) have shown that PMSG is unable to rescue atretic follicles even though sheep follicles, judged atretic by their opacity, vascularity and uniformity of membrana granulosa, when maintained in organ culture revert to seemingly normal follicles and their oocytes,

after transfer to suitably prepared recipients, can produce viable offspring (Moor and Trounson, 1977). In cycling hamsters PMSG increases the number of ovulations by decreasing atresia and recruiting reserve follicles (Chiras and Greenwald, 1978). In the rat PMSG appears to stimulate the growth of all sizes of follicles and prevent atresia (Mauleon and Mariana, 1977). In addition Braw and Tsafiriri (1980) have shown that in the rat PMSG may cause superovulation by rescuing larger follicles from atresia.

#### 2.1.2 Other Gonadotrophic Preparations

In addition to PMSG, defibrinated blood from pregnant mares has been shown to have comparable gonadotrophic activity (Shatalov, 1968).

In domestic livestock, pituitary FSH has been used in an attempt to induce ovulation (reviewed by Betteridge, 1977) although for commercial use its availability in a large enough supply at low enough cost has been questioned (Gosling et al., 1979). Ovine FSH has a half-life of approximately 5 h in both sheep and cattle (Laster, 1972); thus to maintain biologically active levels in blood frequent injections were thought to be necessary. The different methods used to maintain levels have been reviewed by Lopney et al. (1981) who found that, despite the short half-life, a single daily injection of FSH was effective in superovulating beef cattle. In small laboratory animals, such as the rat and rabbit, the half-life is considerably shorter, approximately 1½-2 h (Laster, 1972).

Horse anterior pituitary preparation has also been compared with PMSG (Moore, 1975) and found to be equally effective in producing superovulation in both cows and heifers, but in heifers the extract produced more unruptured follicles than did PMSG. In goats there was a higher rate of fertilization after use of the extract (Moore, 1974).

In humans hMG was first introduced by Steptoe and Edwards (1970) to induce mild superovulation in cycling women, although the ability of postmenopausal urine to induce ovulation in laboratory animals had been described much earlier (Evans and Simpson, 1935), and its ability to stimulate the ovaries of amenorrheal women had been previously described (Borth et al., 1961). Recently several authors (Newcomb, 1980; Critser et al., 1982; Lauria et al., 1982) have shown that, in cattle, hMG can produce superovulatory effects less variable than those found with PMSG, and comparable to those observed after use of FSH.

## 2.2 Induction of Ovulation in Humans

Infertility in humans may be attributed to the male, the female or to both partners. When female infertility has been diagnosed it may then be categorized into one of two broad areas, anovulation or problems subsequent to ovulation. Anovulation may be treated with pharmacological preparations to induce ovulation. In the absence of oviducts or where they are irreversibly blocked,

aspiration of the oocyte from the follicle, in vitro fertilization and subsequent transfer of the embryo to the uterus provide for the possibility of pregnancy in an otherwise infertile woman.

### 2.2.1 Anovulation

Anovulatory women who can be induced to ovulate can be divided into two broad categories: those who have gonadotrophin and oestrogen production but do not cycle, and those who are deficient in gonadotrophins and oestrogens and cannot cycle (Speroff et al., 1978). There are other smaller groups of women who can be treated, including those who have elevated prolactin levels (Hack and Lunenfeld, 1979). Each category has a specific medical therapy for inducing ovulation, based on the endocrine problem involved (Wu and Prazak, 1974).

Greenblatt (1961) first introduced clomiphene citrate (Clomid) to cause the hypothalamus to stimulate the pituitary to augment its secretion of FSH and LH (Speroff et al., 1978), and this has proved an effective treatment for women who produce gonadotrophins and oestrogens but do not cycle. Since its introduction many investigators have reported successful pregnancies following treatment of anovulation with either clomiphene alone or in combination with hCG (e.g., Edwards, 1973; Swyer et al., 1975). On laparoscopy following clomiphene-hCG stimulation it was noted that the percentage of immature oocytes was high when only one follicle with a volume larger than 1 ml was present (Thebault et al., 1982). In contrast, if several large follicles were present one or more oocytes had reached full maturity. These authors therefore suggested that superovulation, even if limited, would mean at

least one oocyte capable of being fertilized could be obtained, and it is therefore ineffective with the present therapeutic procedures to try to stimulate development of a single follicle.

In 1945 Hamblen et al. reported that administration of PMSG and hCG to anovulatory women resulted in ovulation, but it gave inconsistent results which were attributed to the production of antibodies by the women (Lunenfeld, 1963) and its clinical use was abandoned. In 1958 Gemzell et al. reported the successful induction of ovulation with gonadotrophins extracted from human pituitaries, followed by an injection of hCG. Several years later Donini et al. (1964) reported the purification of a gonadotrophin from the urine of post-menopausal women, and in the early 1960's several workers reported successful induction of ovulation and sometimes pregnancy with hMG and hCG (reviewed by Lunenfeld, 1963). Since then gonadotrophins have been extensively used for the induction of ovulation (reviewed by Thompson and Hansen, 1970; Gemzell, 1978; Schwartz and Jewelewicz, 1981).

Pituitary hormones have occasionally been used in an attempt to provide a more physiological ratio of LH and FSH, since it has been suggested that a patient's responsiveness to hMG may be influenced by her endogenous levels of gonadotrophins (Taylor, 1972).

Following induction of ovulation with either clomiphene or hMG, but particularly with hMG, multiple pregnancies have occurred (reviewed by Schenker et al., 1981). With clomiphene most multiple pregnancies have been twins and the abortion rate and incidence of congenital malformations were not increased (Hack et al., 1970; Hack and Lunenfeld, 1979) and birth weights were normal (Human Pituitary

Advisory Committee, 1975). Following treatment with hMG foetal loss due to prematurity in the multiple pregnancies has been a serious problem. As with clomiphene there is a normal incidence of congenital abnormalities and the infants have a normal post-natal development (Hack et al., 1972; Hack and Lunenfeld, 1979).

Thus in the induction of ovulation in anovulatory women much effort has been focused at preventing multiple pregnancies. In addition to the risks of multiple pregnancies, excessive stimulation with gonadotrophins may result in ovarian hyperstimulation syndrome, a potentially fatal condition for women (reviewed by Schenker and Polishuk, 1975; March, 1978; Yaffe et al., 1979).

The serum concentration of oestradiol has been used as an index of follicular maturation during treatment with gonadotrophins (Notation et al., 1978) but there has been some controversy concerning the relationship between preovulatory oestrogen levels and multiple pregnancies. Gemzell and Roos (1966), Johansson and Gemzell (1969) and Muechler et al. (1981) found a relationship between the two although the relationship was not linear, and Taymor et al. (1970) and Karam et al. (1973) suggested that it was possible to prevent the occurrence of triplets or larger pregnancies by withholding hCG if the oestradiol concentrations were too high. In contrast, Brown and Beischer (1972), Wu (1977), Schwartz et al. (1980) and Messinis and Nillius (1981) found no correlation, although monitoring oestradiol levels and withholding hormonal therapy did prevent ovarian hyperstimulation syndrome (Schwartz et al., 1980; Messinis and Nillius, 1981). Gould (1979) concluded that under close medical supervision hormonal stimulation does not have an adverse effect either on the mother or on the offspring.

### 2.2.2 In vitro Fertilization

In contrast to the above situation, in vitro fertilization is indicated in infertile women where tubal blockage is present.

Lopata et al. (1980) have suggested that extracorporeal conception may also be used in women who are unable to conceive because of the production of sperm antibodies, or in some cases of oligospermia.

Since oocyte recovery rates on laparoscopy are fairly low, administration of gonadotrophins to cycling women has been used to increase the number of oocytes maturing (reviewed by Soupart, 1981) and hence increase the probability of successful in vitro fertilization and subsequent pregnancy.

Increasing the yield of oocytes from women was first described by Jagiello et al. (1968) who used FSH to obtain up to 14 oocytes per patient for cytogenetic studies. Steptoe and Edwards (1970) introduced a similar technique in cycling women using hMG, and workers in Australia (Talbot et al., 1976) have used human pituitary gonadotrophin. Clomiphene citrate has also been used to induce stimulated ovarian follicular development in normally cycling women (Lopata et al., 1978a).

In most cases pregnancies to term have not been reported after the transfer of in vitro fertilized embryos into gonadotrophin-primed women (Edwards et al., 1980). With the exception of one patient where a tubal pregnancy ensued (Steptoe and Edwards, 1976) early miscarriages were noted in all the pregnancies observed.

Recently, however, Wortham et al. (1982) have reported a single pregnancy following in vitro fertilization of oocytes classified as



'immature', recovered from gonadotrophin-stimulated women. Edwards (1981) has reviewed the advantages and disadvantages of oocyte collection from stimulated versus natural cycles. As a result of the problems most workers reverted to the collection of a single oocyte during the natural cycle (Lopata et al., 1978b; Edwards et al., 1980; Edwards, 1981).

It was suggested that the pregnancy failure following gonadotrophic stimulation was due to impaired implantation attributed to an insufficient or delayed luteal phase (Edwards et al., 1980). Following stimulation with hMG and hCG, follicular steroid patterns were bizarre (Short, 1979) and the higher the oestrogen concentrations the shorter the luteal phase, with many patients exhibiting only 9-day luteal phases. Aspiration of the follicular fluid and oocyte has been shown either to have no effect on the steroid pattern during the luteal phase (Wrambsy et al., 1981), or to cause a temporary deficiency in plasma progesterone on the third day following aspiration (Frydman et al., 1982). In cynomolgus monkeys after superovulation an inadequate rise of progesterone, indicative of a luteal phase defect has been observed following ovulation (Kreitmann et al., 1982). However, in humans injection of progesterone or hCG during the luteal phase did not correct this abnormality (Edwards and Steptoe, 1975; Edwards et al., 1980). Alternatively, it has been suggested that the pregnancy failure may result from an unbalanced endometrial growth after the drastic rise in oestrogen concentrations after hMG priming (Mandelbaum et al., 1982). Since gonadotrophins and/or clomiphene citrate have been successfully used to induce pregnancies in

anovulatory women the pregnancy failures were unexpected (Short, 1979). It has, however, been claimed that the administration of hMG and hCG to amenorrheal women results in different hormonal patterns compared with those produced by its administration to cycling women, and there is no luteal phase defect in the amenorrheal women (Edwards and Steptoe, 1977). A more recent study (Soules et al., 1980) has suggested that general anaesthesia, as used for oocyte recovery, may inhibit ovarian steroidogenesis and cause problems during the luteal phase.

Recently the use of stimulated cycles for oocyte collection has again been tried and several workers have reported successful pregnancies. Trownson et al. (1981) suggested that the induction of multiple follicles by clomiphene citrate does not induce the endocrine and follicular abnormalities reported by Edwards et al. (1980) and the luteal phase was not reduced in length. In addition, it has been suggested (Trownson et al., 1981; Trownson and Wood, 1981) that controlling the time of oocyte maturation by exogenous hCG may have advantages over the natural cycle, in terms of oocyte collection. Similarly Jones et al. (1982) reported several pregnancies following stimulation with hMG and hCG.

It has been suggested that gonadotrophin treatment of humans may increase the incidence of chromosomally abnormal embryos (Boué and Boué, 1973), but the normality of oocytes recovered after gonadotrophin treatment is difficult to assess, particularly considering that these oocytes receive far more detailed examination than oocytes ovulated during a spontaneous cycle. It has been estimated (Schlesselman, 1979) that as high as 40-50% of human

implanted blastocysts from spontaneous cycles have some chromosomal abnormality. Usually most abnormal embryos die and are eliminated in early pregnancy (Biggers, 1979). In non-human primates it has been suggested (Katzberg and Hendrickx, 1966; Batta et al., 1978) that the use of oocytes from gonadotrophin-treated donors may not be satisfactory because of abnormalities in these oocytes. The authors suggest that such abnormalities could result from asynchrony of ovulation and oocyte maturation.

A maximum number of large follicles and healthy oocytes was obtained when stimulation with hMG occurred around day 8 of the cycle (Mandelbaum et al., 1982). This period of the cycle marks the beginning of atresia of large follicles (greater than 2 mm diameter) adjacent to the one or two preovulatory follicles that will grow until ovulation (Mandelbaum et al., 1982). Gonadotrophin priming at a time when endogenous FSH levels are dropping may counteract this follicular atresia (Gougeon, 1979). In monkeys injection of hMG later than day 8 does not stimulate development of additional follicles even if the dominant follicle is removed (DiZerega and Hodgen, 1980). These authors suggest that this suppression of additional follicular growth may be active or passive, in that the additional follicles merely lose their ability to respond to gonadotrophins. However only 50% of oocytes recovered from monkeys (Kreitmann et al., 1982) or humans (Steptoe and Edwards, 1970) following gonadotrophin stimulation are mature; the remaining oocytes are either immature or atretic. After in vitro insemination with the husband's semen 37% of oocytes from stimulated cycles were fertilized,

as judged by pronuclear formation, compared with 64% from spontaneous cycles (Mettler et al., 1982).

If questions of oocyte normality and fertilizability can be answered, then gonadotrophic stimulation and in vitro fertilization may be a useful technique. Results could be improved by delaying transplantation to a subsequent cycle after embryo freezing, thus avoiding transfer during an hMG-stimulated cycle. A single reported case exists (Jayaraman, 1978) of a human pregnancy resulting from use of this technique, although no details have been published. Even when eggs are collected during spontaneous cycles the delay in development that may occur during in vitro culture may adversely affect synchrony between the uterus and re-implanting embryo (El-Badrawi and Hafez, 1982); this delay would be overcome by embryo freezing.

### 2.3 Induction of Ovulation in Domestic Livestock

The early studies in rats and mice suggested that it might be possible to develop a method for increasing the prolificacy of animals, which would be a valuable technique if it could be applied to certain classes of livestock. However, it is only fairly recently that superovulation, combined with embryo transfer, has been used for proliferating desirable livestock. Details of the regimens and techniques used have been extensively reviewed (Betteridge, 1977; Betteridge and Moore, 1977) together with details of the early history

atteridge, 1981) and will not be covered in this review.

The principle of inducing ovulation or superovulation is similar in most species: a follicle-stimulating gonadotrophin is administered either near the end of the luteal phase of the oestrous cycle, or around the conclusion of treatment with progestagens designed to control the time of ovulation. The degree of superovulation is controlled by varying the dose of gonadotrophin and, in many regimens, prostaglandins have been used to induce luteolysis.

In this review the endocrine response and subsequent fertility following superovulation will be discussed in cattle, sheep and goats.. These livestock are those where superovulation may prove to be most valuable since only small numbers or a single offspring is produced in each breeding season. The potential advantages of superovulation and its possible use combined with embryo transfer have been reviewed (Foote and Onuma, 1970; Gordon, 1975; Seidel, 1981). In addition details of the oestrous cycle and ovarian function in these species have been previously reviewed (Dziuk, 1973; Hafs *et al.*, 1976; Robertson, 1977; Robinson, 1977) and will not be described here.

### 2.3.1 Endocrine Aspects

Some of the studies in domestic livestock have no controls because of factors of cost and convenience, that is, animals which received no hormonal treatments are not included in the studies. Therefore in these studies the conclusions reached are relative to what would be expected to happen in the absence of gonadotrophic

stimulation; this is based on previous studies rather than on what did happen in the particular study being undertaken.

The hormonal profiles following superovulation have been studied by many authors, mainly in cattle. Most workers have measured no LH peaks prior to oestrus (Lemon and Saumande, 1972; Henricks et al., 1973; Hallford et al., 1975; Saumande and Pelletier, 1975; Barbella et al., 1976; Saumande, 1980). However, Spilman et al. (1973) reported a sharp rise in plasma LH concentrations the day after PMSG injection and there is no obvious explanation for this discrepancy. In sheep Evans and Robinson (1980) reported peak LH concentrations prior to the onset of oestrus in ~ 25% of animals, but no details of the time interval were given. Preovulatory LH release was not related to ovulation rate when expressed as maximum plasma LH concentrations (Henricks et al., 1973; Testart et al., 1977; Schams et al., 1978; Sreenan et al., 1978; Evans and Robinson, 1980; Saumande, 1980), suggesting that the increased number of ovulations is due to increased folliculogenesis and that peak LH concentrations are adequate to rupture large numbers of follicles (Evans and Robinson, 1980). Saumande and Pelletier (1975) and Sreenan et al. (1978) found a correlation between ovulation rate and total LH released. In addition, the height of the LH peak was not significantly different from that observed in normal cycling animals (Schams et al., 1978) and was not correlated with the size of the preovulatory oestradiol peak (Henricks et al., 1973; Evans and Robinson, 1980).

Levels of circulating oestrogen rise dramatically before oestrus (Lemon and Saumande, 1972; Henricks et al., 1973; Booth et al.,

1975; Hallford et al., 1975; Saumande and Pelletier, 1975; Testart et al., 1977; Sreenan et al., 1978; Evans and Robinson, 1980; Saumande, 1980; Schneider et al., 1980; Guay and Bedoya, 1981) and at oestrus they may be 4 times as high as in untreated animals (Booth et al., 1975). A highly significant linear increase of peak oestradiol concentrations with dose of PMSG was observed and a higher rate of production of oestrogen by follicles from relatively highly stimulated animals was suggested (Evans and Robinson, 1980). Oestrogen production has been correlated with the ovulation rate (Lemon and Saumande, 1972; Saumande and Pelletier, 1975; Testart et al., 1977; Sreenan et al., 1978; Lopez-Barbella et al., 1979; Evans and Robinson, 1980; Saumande, 1980; Guay and Bedoya, 1981; Saumande and Lopez-Sebastian, 1982). Concentrations of oestrogen in the follicular fluid of superovulated cows have been reported to be the same as those during a normal cycle (Booth et al., 1975).

Postovulatory levels of oestrogen have been reported to remain high (Booth et al., 1975; Kummer et al., 1980), or to be high only on sporadic occasions (Saumande, 1978) and they have been correlated with the number of large follicles present at this time (Kummer et al., 1980; Guay and Bedoya, 1981), although other workers have been unable to find such a correlation (Testart et al., 1977).

Peak oestradiol levels are much higher in the superovulated calf compared to the superovulated cow, and the authors have suggested that this is either related to the larger blood volume in adult animals, or to more large unovulated follicles in the calf (Testart et al., 1977).

Since both sheep and rat follicles have been shown to lose their ability to synthesize oestrogen after exposure to preovulatory gonadotrophins (Hori et al., 1970; Moor, 1974; Lieberman et al., 1975) it has been suggested that postovulatory oestrogen must come from fresh follicular growth, resulting from continued PMSG stimulation (Saumande, 1978).

Progesterone levels often rise extremely high after superovulation (Spilman et al., 1973; Booth et al., 1975; Agthe et al., 1976; Saumande, 1978; Schams et al., 1978; Schneider et al., 1980) and have been correlated with the numbers of ovulations (Bindon et al., 1971; Lamond and Gaddy, 1972; Agthe et al., 1976; Eastwood et al., 1976; Testart et al., 1977; Evans and Robinson, 1980; Riedel et al., 1980; Guay and Bedoya 1981). Other authors have been unable to show any such correlation (Rajamahendran et al., 1976; Schams et al., 1978; Solti et al., 1978) and there is no obvious explanation for this discrepancy. Elsaessar et al. (1981) have shown a correlation between concentrations of milk progesterone and the numbers of ovulations.

Booth et al. (1975) have demonstrated a negative correlation of progesterone levels with day 6 embryo recovery in cattle. Betteridge et al. (1982) were unable to correlate progesterone levels in cattle with the size of embryos on day 13, which is opposite to the situation in sheep, where Wintenberger-Torres (1968) has suggested that elevated progesterone levels cause accelerated growth after day 8.

As a consequence of the extremely high levels of circulating progesterone the length of the oestrous cycle is frequently



extended in nonpregnant animals (Spilman et al., 1973).

Progesterone concentrations in peripheral plasma have been shown to be significantly higher after superovulation with PMSG compared with FSH, when a comparable number of corpora lutea are present (Schams et al., 1979). In a study of the endocrine responses of goats superovulated with PMSG or FSH Armstrong et al. (1983) observed that the oestrogenic response to FSH was considerably less than to PMSG. The greater response in the PMSG-treated group was attributed to the higher incidence of large follicles which failed to ovulate.

#### 2.3.2 Fertility following Superovulation

Results regarding fertilization and subsequent embryo viability following superovulation in domestic livestock are often difficult to compare because of the different criteria used to assess fertility. In addition many studies do not include control animals which ovulated spontaneously and consequently losses due to technical procedures are difficult to assess. Even in the absence of gonadotrophic stimulation embryonic mortality in cattle breeding programs has been estimated at ~ 30% (Sreenan et al., 1979). Furthermore, following superovulation it has been shown that insemination can affect fertility, both in terms of the routine used and the quality of the semen (Moore and Eppleston, 1979; Newcomb, 1980). An additional factor is the variability of eggs of the same stage put in the same in vitro culture conditions, but coming from different donors (Trounson et al., 1976). Thus care must be taken in assessing fertility following superovulation.

It has been reported that in cattle the proportion of oocytes fertilized decreases as the ovulation rate increases (Scanlon, 1968; Laster, 1973; Shea et al., 1976), whereas Boland et al. (1978b) found no such decrease. In sheep increasing doses of PMSG give rise to a decreased percentage of oocytes fertilized (Mutiga and Baker, 1982). Other authors have reported low fertilization rates after superovulation without quantitatively studying the relationship between ovulation rate and fertilization (Onuma et al., 1970). Rowson (1971) has suggested that the LH-like activity of the PMSG may cause some premature ovulations, and the progesterone from these newly formed corpora lutea might then adversely affect subsequent fertilization. In ewes, irrespective of the type of superovulatory treatment fertilization frequently fails (Betteridge and Moore, 1977) and this failure appears to be due to faulty transport of spermatozoa through the cervix (Trounson and Moore, 1974). Prepubertal lambs can also be superovulated (Mansour, 1959) but the fertilization rates are low after mating and, as with the adult, surgical insemination may be required to obtain fertilization. Moore and Eppleston (1979) obtained good ovulation rates in a small series of PMSG-treated Angora goats, but only 46% of ovulated eggs were fertilized, largely due to complete fertilization failure in several animals. Fertilization rates were improved to ~ 70% by use of a crude equine pituitary extract (Moore, 1974). Similar results were obtained by Sugie et al. (1982) who found that 63% of oocytes recovered from superovulated goats were fertilized, but no fertilized oocytes were recovered from 28% of animals. The same authors found that only 21% of oocytes recovered

from superovulated kids were fertilized. Robinson (1951a) attributed most of the failure of fertilization in sheep to a faulty timing between ovulation and insemination.

In cattle (Casida et al., 1943; Black et al., 1953; Jainudeen et al., 1966; Foote and Onuma, 1970; Gordon, 1975), sheep (Mansour, 1959; Wright et al., 1976) and goats (Sugie et al., 1982) the prepubertal animal responds readily to gonadotrophic stimulation, but fertility and egg recovery rates are low. Development from the 8-cell to blastocyst stage appears to be impaired in embryos collected from superovulated lambs (Wright et al., 1976). This may not be related to the superovulatory treatment, since in lambs induction of puberty with low doses of PMSG results in a high fertilization rate (> 90%), which is not related to the number of corpora lutea (1-4), but less than 50% conception rate (Quirke, 1981). Robinson (1951a,b), Bindon et al. (1971) and Quirke (1979) have estimated that most of these embryonic losses occur within the first 4 weeks of pregnancy. Similarly, transfer of 8- to 16-cell embryos from ewe lambs to adult recipients has shown that these embryos have less than half the developmental capacity of embryos transferred from adult ewes (Quirke and Hanrahan, 1977); this decreased potential either is inherent to the oocyte, or results from the time spent in reproductive tract of the ewe lamb.

Sperm transport may be a problem in calves, where fertilization can be obtained if semen is introduced directly into the uterus. (Gordon, 1975). Howe and Black (1963) have shown that transport of sperm does occur in prepubertal calves treated with PMSG,

but no quantitative data were given. In calves a low rate of egg recovery has been attributed to the effect of endogenous oestrogen on egg transport, the trapping of some oocytes within the follicles or losses due to the immature fimbriae being too small to surround the ovary thus causing failure of pick-up (Hafez, 1969).

From in vitro studies, Onuma and Foote (1969) concluded that the developmental potential of oocytes from calves was equal to that from sexually mature cows whereas, from endocrinological and histological studies, Saumande (1978) has concluded that eggs from prepubertal calves are of poor quality, even though follicular growth is good. He attributes this either to the deleterious effects of high steroid concentrations, these being higher than the adult for the same number of ovulations, or to a lack of some essential maturation factor. Seidel et al. (1971a,b) suggested that prolonged residence of embryos in the reproductive tract of calves reduced their viability.

Many authors have not looked at fertilization following superovulation, but rather at the proportion of degenerate or abnormal eggs at a variety of times post coitus. As the ovulation rate increases the proportion of degenerate eggs also increases (Gordon, 1975; Church and Shea, 1977; Boland et al., 1978a,b; Greve et al., 1979; Rhind et al., 1980). Other evidence suggests that there is great variability between individual animals, irrespective of the number of eggs ovulated (reviewed by du Mesnil du Buisson et al., 1977). This may be a direct consequence of the stimulation of a large number of follicles which do not ovulate, but cause high oestrogen levels during follicular stimulation and immediately after ovulation

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(Booth et al., 1975), but this too has been questioned (du Mesnil du Buisson et al., 1977; Saumande, 1978). Since it has been suggested that, after the preovulatory release of gonadotrophin, all follicles greater than ~ 3 mm luteinize or undergo atresia (Arrau, 1974) and such follicles are unable to secrete oestradiol (Hay and Moor, 1975), Saumande (1978) has suggested that any postovulatory oestradiol must come from follicles stimulated to grow as a result of the long-lasting action of PMSG.

While numbers of embryos recovered do not appear to change with time after ovulation (Betteridge, 1977) most authors report a progressive decline in embryo quality, which occurs in cattle mainly between days 3 and 8 (Seidel et al., 1971; Newcomb et al., 1976; Boland et al., 1978a; Elsdon et al., 1978; Renard and Heyman, 1979). In sheep increased embryonic mortality has been reported between days 14-16 (Wintenberger-Torres, 1968) which the author suggests is due to an increased embryonic sensitivity to elevated progesterone levels following superovulation (Wintenberger-Torres and Rombauts, 1968). A similar peak in mortality was observed several years earlier by Robinson (1951b).

It has often been assumed that the unusual endocrine environment following superovulation must adversely affect embryo recovery rates and viability (Betteridge, 1977). Rowson (1971) has suggested that in cattle, when more than 20 ovulations are present, the physiology of the reproductive tract is disturbed. Henricks et al. (1973) have proposed that, following superovulation, progesterone production continues beyond the usual time, giving rise to an hormonal

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environment adverse to fertilization. Recovery of superovulated eggs from cattle on days 3-4 followed by 2-3 days' storage in the oviduct of a rabbit improved transfer results on days 5-6, compared with a direct transfer (Boland et al., 1978b); this could however be related to the more intense screening process undergone by eggs incubated in the rabbit. When the uterotubular junction of superovulated heifers was ligated on day 3 normally developing eggs were recovered from the oviducts on days 7-8, whereas flushing tubal eggs towards the uterus on day 3 caused the majority of eggs to degenerate (Newcomb et al., 1976).

Elsden et al. (1978) found that, on transfer, pregnancy rates from untreated donors were higher than from superovulated donors although all embryos appeared normal at the time of transfer. They suggested that exogenous gonadotrophins may damage some of the embryos by producing abnormal steroid patterns, but that the harmful effects may only become obvious as the embryo ages.

Several authors have observed premature regression of the corpora lutea in superovulated donors. This has been reported in cows (Onuma et al., 1970; Bellows and Short, 1972; Bouters et al., 1980; Betteridge et al., 1982), sheep (Sreenan, 1978; Willadsen, 1978) and goats (Armstrong et al., 1982a) following superovulation with PMSG.

Comparing PMSG and FSH, the use of FSH has been shown to result in a much lower incidence of luteal failure in goats (Armstrong et al., 1982b) and, in cattle, for a comparable number of corpora lutea and recovered oocytes the percentage of embryos at the expected

stage of development and showing no signs of degeneration is significantly higher (Critser et al., 1980).

Several authors have suggested that superovulation may result in accelerated embryo transport (Robinson, 1951a,b; Rowson, 1951; Whyman and Moore, 1980). Rowson (1951) and Avery and Graham (1962) have attributed the increased rate of transport to progesterone from the corpora lutea existing prior to superovulation, since the problem was exacerbated in animals with such corpora lutea. Whyman and Moore (1980) have suggested that PMSG decreased the proportion of eggs fertilized and accelerated embryo transport, while Robinson (1951a) has suggested that the two are interrelated. Newcomb et al. (1976) have shown that premature entry of the embryos into the uterus is detrimental to survival, at least in cattle.

## 2.4 Studies in the Rat

### 2.4.1 Oestrous Cycle and Pregnancy

A number of factors such as cost, ease of handling and the short time span of generations, coupled with large litters have contributed to the value of the rat as an experimental animal. As a result many aspects of reproduction have been studied in great detail in the laboratory rat, and it has frequently been used as a model for the manipulation of events involved in reproduction.

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In 1922 Long and Evans published an extensive study of the reproductive life of the female rat and most of their observations are still valid today. One major difference, however, is that modern breeding techniques have lowered the age of the first ovulation from a mean of 77 days (Long and Evans, 1922) to 35-40 days (Ramaley, 1979; Yamamoto et al., 1980). Following puberty the animal may be considered to have reached sexual maturity, the earliest stage at which it is capable of producing young. Thereafter a pattern of hormonal and follicular changes, referred to as the oestrous cycle, is repeated every 4 or 5 days (Long and Evans, 1922). The term 'oestrus' refers to periods of sexual receptivity and excitability observed in many animals (Heape, 1900), and the different stages of the cycle in the rat are characterized by histological changes in the epithelium of the uterus and vagina (Long and Evans, 1922) and by changes in the profiles of serum hormones (Brown-Grant et al., 1970; Butcher et al., 1974a; Kalra and Kalra, 1974; Smith et al., 1975; Nequin et al., 1979; Belanger et al., 1981). The mechanisms regulating the oestrous cycle involve the hypothalamic-pituitary-ovarian axis and have been extensively reviewed (Everett, 1961, 1964).

2. During the period of oestrus ovulation occurs and the control of ovulation in the rat has also been well reviewed (Schwartz, 1973). The rat is classified as a spontaneous ovulator, since on the day preceding oestrus (prooestrus) it releases an ovulatory surge of LH and ovulation subsequently occurs at approximately 0200 h on the morning of oestrus, even in the absence of mating (Schwartz, 1973).

During maturation the primary oocyte and its surrounding



cells undergo a series of changes in which the follicle becomes multilaminar, with a fluid-filled antrum. The oocyte completes its first meiotic division and the first polar body is extruded (reviewed by Hertig and Barton, 1973). Recently ovulated oocytes remain surrounded by the corona radiata cells as they lie within the oviducts (Odor, 1960).

The pre-implantation period of the embryo's life includes a number of important developmental and biochemical events, all of which have been studied in great detail. One of the earliest events is fertilization (reviewed by Austin, 1965; Gwatkin, 1977), during which a sequence of changes occurs which culminates in the fusion of the sperm head with the nucleus of the oocyte (reviewed by Blandau, 1961). This is followed by cleavage of the zygote in the oviduct. Transport of normal fertilized eggs through the oviducts takes approximately 90 h and on the fourth day they enter the uterus (Psychoyos, 1967). From days 4-5 the eggs lie free in the uterine cavity and during this time they undergo cavitation to form blastocysts (Psychoyos, 1973b). In the afternoon of day 5 the zona pellucida is lost, presumably by a lytic process whose mechanism is unclear (Dickman and Noyes, 1961; Dickman, 1969), but which appears to involve an oestrogen-dependent proteolytic enzyme of endometrial origin (Joshi and Murray, 1974; Rosenfeld and Joshi, 1977). Implantation is then initiated (reviewed by Psychoyos 1973a,b). Following implantation foetal growth continues and the hormonal changes during the postimplantation phase of pregnancy and parturition have been extensively reviewed (Heap et al., 1973). Parturition in the adult

rat occurs approximately 22 days post ovulation (Farris, 1962; Zarrow et al., 1969a).

#### 2.4.2 Induction of Ovulation

In the earliest report of induction of ovulation in rats daily intramuscular transplants of anterior pituitary tissue resulted in a large number of ovulations (P.E. Smith, 1926; Engle, 1927; Smith and Engle, 1927). The method of inducing ovulation was simplified by Cole and Hart (1930) who observed that the serum of pregnant mares could produce superovulation in immature rats. Further studies by Cole et al. (1932) showed that repeated injections of pregnant mares' serum were no more effective than a single dosage and that excessively large doses gave rise to corpora lutea atretica, rather than large numbers of ovulations. Rowlands (1944) and Rowlands and Williams (1944) looked at the ability of mare serum gonadotrophin to induce ovulation in both intact and hypophysectomized immature rats. They concluded that, although the mare serum gonadotrophin was effective in producing follicular growth, it had only limited capacity to produce ovulation and an injection of chorionic gonadotrophin 48-72 h after the initial injection proved to be effective in producing ovulation. When the dose of serum preparation was tripled, superovulation was produced, but further increase caused overstimulation which prevented ovulation.

More recently it has been shown that ovulation can be induced in rats as young as 25 days of age by a single injection of PMSG on day 22 (McCormack and Meyer, 1962), or as young as 23 days

of age with a single injection of hCG on day 20 (Sugawara et al., 1969; Sugawara and Takeuchi, 1970). PMSG and hCG in combination can produce ovulation in 17- to 18-day-old rats (Zarrow and Wilson, 1961) although, prior to day 21, not all rats respond to the gonadotrophic stimulus.

McCormack and Meyer (1962) and Zarrow and Quinn (1963) apparently independently recognized that immature rats ovulate in response to a single injection of PMSG and that hCG is unnecessary for the release of oocytes from properly primed follicles. In general, any given dose of PMSG stimulates the secretion of a quantity of endogenous LH sufficient to rupture the majority of competent follicles (Coppola et al., 1966). There has been some controversy over the need for hCG. The earlier reports of Cole (1936) and Cartland and Nelson (1938) indicated that PMSG alone could induce ovulation in the rat, whereas Rowlands (1944) and later workers (Lunn and Bell, 1968; Peluso et al., 1977, 1980) found that PMSG alone was usually ineffective. Zarrow and Quinn (1963) and Zarrow and Brown-Grant (1974) have shown that immature rats respond to PMSG and hCG over a wider age span than to PMSG alone; the discrepancies reported previously may reflect the use of rats at an age when they are not able to respond to a single injection of PMSG. In addition, the dose response curve for PMSG-induced ovulations appears to be complex; there is a triphasic response, with doses of 10-20 i.u. showing no response (Ying and Meyer, 1969a) and excessively high doses (> 50 i.u.) causing a marked decrease in the number of ovulations and formation of cystic follicles (Rowlands, 1944; Wilson and Zarrow, 1962). The optimum dose for the

induction of ovulation in the rat also appears to be dependent on the age (Zarrow and Wilson, 1961; Zarrow and Quinn, 1963; Zarrow and Brown-Grant, 1974) and even on the strain (Wilson et al., 1974).

Strauss and Meyer (1962) described a simple procedure for inducing synchronous ovulation in ~ 90% of prepubertal rats. These authors injected 8 i.u. PMSG s.c. into 30-day-old female rats and found that ovulation occurred early in the morning of day 33. This procedure was further studied by Nuti et al. (1975) who investigated if the immature rat, synchronized to ovulate, was a suitable model for adult animals in studies of reproduction. These authors found that PMSG-treated immature rats could conceive and maintain viable foetuses to term and that hormone profiles were similar to those reported by other workers in adult pregnant rats. In a later study Yamamoto et al. (1978) observed that a minimum dose of 3 i.u. PMSG was required to induce ovulation reliably in immature rats.

The sequence of events from PMSG injection to ovulation is generally considered to be as follows: in response to the FSH activity of PMSG several follicles mature and produce oestrogen, the level of which rises in the circulation until a 'critical period' in the afternoon of the second day after PMSG. The hypothalamus is stimulated to produce LHRH which activates the anterior pituitary to release LH. Shortly thereafter meiosis resumes within the oocytes of the matured Graafian follicles and ovulation occurs several hours later (Gates and Bozarth, 1978). Both McCormack and Meyer (1962) and Zarrow and Quinn (1963) have shown that ovulation in the PMSG-primed immature rat is due to release of 'ovulating hormone' from the animal's

own pituitary, with the possibility of a progesterone-dependent step being involved (Kohda et al., 1980). This preovulatory surge of gonadotrophin occurs 54-56 h after PMSG (McCormack and Meyer, 1962; Zarrow and Brown-Grant, 1964) and its magnitude is unrelated to the number of oocytes shed (Klawon et al., 1971), although under some conditions the number of oocytes shed appears to be a function of the amount of LH available (Zarrow et al., 1958). Anterior hypothalamic lesions (Quinn and Zarrow, 1964) or carefully timed administration of barbiturates (Strauss and Meyer, 1962) block the PMSG-induced ovulation in immature rats. In very young rats progesterone appears to facilitate ovulation (McCormack and Meyer, 1963). The number of eggs to be ovulated after stimulation with PMSG is not fixed for at least 24 h, since administration of oestrogen at this time can influence the number (Wyss and Pincus, 1964).

Early attempts to produce superovulation in adult rats proved less successful than the studies with the immature animal. Cole (1937) found that small doses of the gonadotrophic extract of pregnant mares' serum given during metoestrus increased the average litter size slightly, but no litter exceeded in size the largest found in control animals. Much later Miyamoto and Chang (1973) induced superovulation in adult animals with an i.p. injection of 30 i.u. PMSG on the morning of oestrus, followed by hCG 52-58 h later. A large number of oocytes were recovered from the oviducts 14-16 h after the injection of hCG.

Superovulation has also been produced with a number of other gonadotrophic substances, such as a combination of pregnancy

and menopausal urine administered to hypophysectomized animals (Leonard and Smith, 1933) and administration of menopausal urine alone to intact immature rats (Evans and Simpson, 1935). In 1940 Evans and Simpson injected a crude FSH preparation into immature rats and found that this would produce superovulation. Similarly, Carter et al. (1958) found that daily injection of a crude FSH preparation into hypophysectomized rats would also induce ovulation. In addition to gonadotrophic preparations both oestradiol (Ramirez and Sawyer, 1965) and testosterone (Zarrow et al., 1969b) can advance puberty in the rat.

#### 2.4.3 Induced Ovulation and Fertility

Most of the early studies with transplants of anterior pituitary tissue (P.E. Smith, 1926; Engle, 1927; Smith and Engle, 1927) looked only at ovulation and not at later pregnancy, but Engle (1927) found 19-29 implantation sites in adult mice given such transplants. However, in similarly treated immature animals there was never any evidence of implantation, although morulae and blastocysts were recovered from the reproductive tracts. Cole (1936) showed that at least some immature rats induced to ovulate with mare serum gonadotrophin were capable of producing live young, and hence he concluded that a true sexual maturity had been obtained. This contrasts with the work of Engle (1931) who concluded that in mice pituitary implants did not induce a true sexual maturity, since the animals did not continue to cycle, nor was pregnancy maintained. In a later study in immature rats Cole (1937) found that superovulation was followed by implantation of a number of fetuses (2-28) far

exceeding that found in the normal mature female. He also found that relatively low doses of the gonadotrophic hormone resulted in as many as 27 fetuses on days 17-22 after mating, although a large number of these were judged to be abnormal. When the dose of hormone was increased twofold the proportion of rats pregnant decreased dramatically, leading him to conclude that the percentage of fertile matings was inversely proportional to the amount of hormone administered. Cole (1940) later reported a maximum of 33 fetuses at midgestation and 23 live young born. From his many studies he concluded that foetal resorption was evidently responsible for the large reduction which occurred during the latter part of gestation.

Evans and Simpson (1940) found similar results using a single injection of follicle-stimulating pituitary extract to induce ovulation in immature rats. Again it was shown that progressively higher doses were less successful in producing increased numbers of implantation sites. The largest number of live young born was 17 and the authors stressed that foetal resorption was an important factor in limiting the number of live young born. Evans and Simpson (1940) concluded that, although superovulation could result in the implantation of many blastocysts, the number of young born rarely exceeded the normal and frequently very few or no young were born. Gestation also tended to be prolonged.

The efficiency of the reproductive processes initiated by these early studies was poor and, although foetal resorption was an important source of loss, large losses also occurred prior to implantation. Both Cole (1937) and Evans and Simpson (1940) observed,

a low percentage of rats mating and of those rats which did mate only 70-80% had evidence of implantation at autopsy. After treating immature mice with anterior pituitary extract Engle, (1931) found that not all animals which mated had ovulated. In addition, he observed a considerable discrepancy between the number of oocytes recovered and the number of corpora lutea. No implantation sites were observed, but this was not due solely to inadequate maturation of the endometrium because it was possible to obtain a decidual response. Engle (1931) considered that failure of ovulation, fertilization and implantation were all causes of low fecundity, but the relative importance of each was not discussed. Also in mice, Bowman and Roberts (1958) noted that the probability of implantation decreased as the number of oocytes shed increased. Wilson and Edwards (1963) observed that, following superovulation of adult mice, the response was variable with more than 50% of animals having no implantation sites, whereas in other animals the litter size was increased. McLaren and Michie (1959) carried out a comprehensive study of pregnancy following superovulation in the mouse, and from their results they concluded that the incidence of early post-implantation death was not related to the number of embryos implanting, whereas late foetal death was markedly dependent on intrauterine crowding.

Austin (1950) attempted to provide quantitative data on the losses following superovulation in immature rats. He found losses at all stages leading to implantation, but the major losses were due to a low rate of mating, 'incomplete stage of oestrus', and a low



rate of fertilization. In addition fragmentation frequently occurred, which was attributed to apparent abnormalities in the eggs. Failure of ovulation and implantation were considered of minor significance.

Evans and Simpson (1940) first suspected the occurrence of delayed implantation in prepubertal rats treated with FSH because gestation was prolonged in some instances up to 31 days. In a later study Zarrow et al. (1969a) reported that the prolonged gestation in prepubertal rats injected with PMSG was due to a delay in parturition, rather than to a delay in implantation. A deficiency in oestrogen or an imbalance of oestrogen to progesterone has been suggested as a possible cause of delayed implantation in weanling rats treated with PMSG and ovarian steroids (Khan and Meyer, 1969). More recently it has been shown that superovulatory doses of PMSG can result in delayed implantation (16 i.u.) or infertility (40 i.u.) in immature rats (Miller and Armstrong, 1981a,b). Delay of implantation has been observed with doses as low as 8 i.u. and the day of injection appears to modify the response (Wu, 1976).

#### 2.4.4 Endocrine Aspects and the Role of Oestrogen

Injected PMSG not only directly stimulates follicular development and maturation at the ovarian level, but also by increasing ovarian steroid secretion indirectly induces the release of endogenous gonadotrophins at the ~~hypothalamic~~-pituitary level (Strauss and Meyer, 1962; McCormack and Meyer, 1963; Ying and Meyer, 1972). Under the influence of graded doses of gonadotrophin the ovary will develop to different degrees and consequently secrete different amounts of

oestrogen and progesterone (Wilson et al., 1974), and the effects of these steroids on the hypothalamic-pituitary complex and on the uterus will therefore also be altered.

The role of oestrogens in infertility or reduced fertility is complex and varies between species (Greenwald, 1967). M.G. Smith (1926) first showed that in adult rats, injection of ovarian follicular extracts on days 1-5 after ovulation resulted in a failure of the pregnancy. Later it was shown that administration of oestrone after implantation caused embryonic death (Huggett and Pritchard, 1945). In addition, at least in the rabbit, alterations in the level of circulating oestradiol may affect the transport of spermatozoa (Noyes, 1959). After ovulation oestrogens may therefore act at different times and locations to cause a failure of pregnancy; this may in part relate to the presence of specific oestradiol receptors in both the oviduct and uterus (Sen and Talwar, 1973).

Administration of oestrogens to female rats on day 1 of pregnancy (Greenwald, 1961; Banik and Pincus, 1964; Ortiz et al., 1979) or transport of oestrogen to the female by insemination with semen from a male pretreated with oestradiol (Banerjee, 1968) accelerates the transport of the zygotes, with a latency of several hours. The zygotes pass prematurely from the oviduct into the uterus and are subsequently expelled through the vagina (Ortiz et al., 1979), a loss which cannot be counteracted by administration of progesterone (Banik and Pincus, 1964). Eggs not accelerated are presumed to be transported at the normal time, since implantation of a number of embryos equal to the number remaining in the tract after the expulsion

phase is found to occur (Ortiz et al., 1979). Although a direct action of oestrogen on the uterus cannot be ignored, it appears that eggs arriving early in the uterus, as is the case following early transfer, are usually expelled (Dickman and Noyes, 1960). A similar expulsion of young embryos arriving early in the uterus has been demonstrated in the rabbit (Adams, 1979).

It has also been suggested that oestradiol is detrimental to blastocyst survival, since blastocysts transferred to the uteri of cycling rats immediately after ovariectomy did not survive, whereas transfer after some days of progesterone injection resulted in survival (Dickman, 1967). This has been interpreted as progesterone neutralizing the uterine effects of oestradiol which were harmful to blastocysts.

Oestradiol can also detrimentally affect implantation. Kincl and Dorfman (1965) showed that inhibition of implantation was related to the dose of oestrogen and that, at intermediate doses, a reduced number of implantation sites was observed. Emmens and Finn (1962) showed that local injection of oestradiol on day 4 not only inhibited implantation and terminated pregnancy in that horn, but also in the contralateral horn.

In addition to postovulatory effects it has been suggested that prolonged exposure of the oocyte to high preovulatory levels of oestrogen may also be detrimental to subsequent embryo development. Most of these studies have used a delay in ovulation to prolong exposure of the intrafollicular oocyte to oestradiol, and subsequently result in abnormal embryo development in both the adult

(Fugo and Butcher, 1966; Butcher et al., 1969b, 1974b; Butcher and Pope, 1979) and immature rat (Martin and Terranova, 1982). The mechanism by which oestrogen induces abnormal embryonic development is unknown, but it has been related to alterations both in the oocyte prior to fertilization (Butcher and Fugo, 1967; Peluso and Butcher, 1974a,b) and in the intrauterine environment during implantation and later foetal development (Butcher et al., 1969a,b). This is in addition to increased abnormalities during fertilization and early cleavage, observed by some authors (Fugo and Butcher, 1966) but not others (Toyoda and Chang, 1969). In superovulated mice injection of oestradiol at the time of hCG administration had no effect on the incidence of polyspermy nor was there any detectable acceleration of oocyte transport (Fraser and Maudlin, 1979); later development was not, however, assessed. During the period of delayed ovulation the oocyte remains at a constant size and in meiotic arrest while the follicle continues to enlarge (Freeman et al., 1970). Ultra-structural analyses have demonstrated changes in the number of cortical granules, which may explain the increase in polyspermy observed in eggs aged within the follicle, and there are also changes in the mitochondrial morphology which suggest alterations in metabolism (Peluso and Butcher, 1974b). Serum levels of oestradiol remain elevated during delayed ovulation (Butcher et al., 1974b; Terranova and Ascanio, 1982) and it has even been suggested that the preovulatory follicles may actually be producing more oestradiol during the period of delay because of a phenobarbital-induced increase in oestradiol metabolism (Martin and Terranova, 1982).

#### 2.4.5 Normality of Oocytes

The normality of superovulated rat oocytes has not been adequately assessed, but in other species data on genetic abnormalities induced by superovulation are inconclusive and contradictory. Several authors have reported no increase in genetic or developmental defects in young derived from superovulated oocytes of humans (Hack and Lunenfeld, 1979), hamsters (Fleming and Yanagimachi, 1980) and rabbits (Maurer et al., 1968). This does not, however, preclude the possibility that defective embryos or foetuses were eliminated earlier in the pregnancy.

In mice, Gates (1965) has suggested that the oocytes produced from superovulated donors are genetically normal, as assessed by in vitro development from the 2-cell to blastocyst stage; these blastocysts gave rise to viable foetuses when transferred to suitable recipients. However since no assessment was made of the number of oocytes ovulated by each animal the 2-cell embryos used in his studies may well have been taken from a population which had already been subjected to selection. Beaumont and Smith (1975) carried out a quantitative study of embryonic losses during both the pre- and post-implantation stages of pregnancy in the mouse. By day 2 a larger proportion of zygotes from superovulated donors were classified as abnormal, but from the design of their experiment they were unable to ascertain whether this was due to abnormalities in the oocyte or to defects in fertilization and early development. In mice and rabbits there is conflicting evidence regarding the chromosomal normality of superovulated oocytes. Some authors have suggested that there is a

significant increase in abnormalities (Fujimoto et al., 1974; Tagaki and Sasaki, 1977), whereas other authors claim there is no increase (Fechheimer and Beatty, 1974; Gray and Chrisman, 1980). Still other authors (Maudlin and Fraser, 1977) have only observed increased polyspermy following in vitro fertilization, but this has subsequently been related to the sperm concentration (Fraser and Maudlin, 1978). In the rat, Miyamoto and Chang (1973) have reported that the rate of fertilization of oocytes recovered from superovulated donors was lower than from naturally ovulating donors, when assessed in vitro.

### CHAPTER 3

#### RATIONALE AND OBJECTIVES

The number of mature oocytes developing to term fetuses is frequently reduced in humans, domestic livestock and small laboratory animals after superovulation with exogenous gonadotrophins (Chapter 2). Under some circumstances the total number of offspring may be increased despite increased embryo losses, whereas in other cases a decreased number of offspring or complete loss of pregnancy may result. Defects resulting in infertility could occur in any of the processes from oocyte maturation through ovulation, fertilization, early cleavage of the embryo and implantation, to later foetal development. Alternatively there could be defects in the oviductal or uterine environments or multiple defects could occur.

The immature rat has been shown to be a good model for the study of the events occurring at ovulation and during pregnancy, in the rat. In addition, many aspects of reproduction have been studied in great detail in this species. The research presented in this thesis uses the immature rat as a model for the infertility following superovulation with exogenous gonadotrophins. Several aspects of the pregnancy were therefore studied in the superovulated immature rat, with the aim of furthering the understanding of the factors involved in this infertility.

The use of PMSG to induce ovulation or superovulation is widespread, both in domestic livestock and laboratory mammals. However,

in most species studied PMSG has been shown to have a half-life greater than 20 h (Chapter 2) and many of the undesirable side effects associated with its use have been attributed to this long half-life.

In an attempt to overcome some of these undesirable side effects, an antiserum to PMSG was used to shorten the period of action of the PMSG. Chapter 5 describes the characterization of this antiserum and in Chapter 6 its use to limit the period of action of PMSG is described. Further studies using this antiserum are described in Chapter 8, where results of studies on implantation in superovulated antiserum-treated rats are reported. Chapter 6 also compares the use of PMSG and FSH for the induction of ovulation and subsequent pregnancy in the immature rat and in Chapter 7 some studies on the pre-implantation stages of pregnancy in the PMSG-superovulated rat are described.

Thus the aims of the studies presented in this thesis were:

- 1) to determine if the period of action of PMSG could be limited by the use of an antiserum, and to investigate the effect of such a limitation on the subsequent pregnancy;
- 2) to compare fertility following superovulation with either PMSG or FSH;
- 3) to investigate the time course of ovulation in the superovulated rat;
- 4) to determine the normality of oocytes produced by superovulation, by transfer of the oocytes immediately after ovulation, to 'physiologically normal' recipients;
- 5) to investigate fertilization in vivo after superovulation;



- 6) to continue earlier studies on the role of oestrogen in the infertility seen after superovulation, by use of both inhibitors of oestradiol synthesis and a specific oestradiol antiserum;
- 7) to investigate the normality of the blastocyst and the uterine environment at the time of implantation, in superovulated rats, using the techniques of blastocyst transfer and traumatization of the uterus to produce decidualization.

## CHAPTER 4

### GENERAL MATERIALS AND METHODS

#### 4.1 Animals

All rats were purchased from Charles River Canada Inc. (formerly Canadian Breeding Farm and Laboratories), St. Constant, Quebec. With the exception of adult Long-Evans rats used in Chapter 7 all rats were of the Sprague-Dawley strain. Immature rats were obtained at body weight 45-50 g and were kept until they weighed 70-80 g (approximately 1 week), which was designated as day -2, and treatments were commenced (approximate age 29 days). In subsequent chapters immature rats refer to those of body weight 70-80 g. Adult rats were purchased at body weight 100-120 g and were kept for at least 1 week prior to experiments, to allow the animals to adjust to their new environment.

All rats were exposed to a 14 h light, 10 h dark cycle with the lights on from 0500 h to 1900 h, unless otherwise indicated (Chapter 7). Room temperature was controlled (22°C) and all animals were allowed free access to food and water.

## 4.2 Experimental Protocol.

In all experiments immature rats were randomly allocated to treatment groups. At 0800-0900 h on day -2, rats were injected s.c. with either 4 i.u. PMSG (control) or 40 i.u. PMSG (SOV). Details of additional treatments are given in subsequent chapters. Unless indicated, all rats were caged on the evening of day 0 with male rats of proven fertility. Mating was judged to have occurred by the presence of a vaginal plug and/or the presence of spermatozoa in a vaginal smear on the following morning (day 1). Only animals which mated continued to be part of the experiments.

## 4.3 Hormones and Drugs

The following hormones and drugs were used in this study:

### Hormones

PMSG (Equinex)	Ayerst Laboratories; Montreal, P.Q.
progesterone	Sigma Chemical Co., St. Louis, MO.
hCG	Sigma Chemical Co., St. Louis, MO.
oestradiol	Steraloids Inc., Wilton, NH.
FSH	gift from Dr. D. T. Armstrong
LH	National Pituitary Agency, Bethesda, MD.

### Antisera

rat LH a/s                      gift from Dr. C. S. Sheela Rani  
PMSG a/s                        gift from Dr. D. T. Armstrong

### Drugs.

[Des-Gly<sup>10</sup>,D-Ala<sup>6</sup>,Pro-NHEt<sup>9</sup>]LHRH

Sigma Chemical Co., St. Louis, MO.

4-acetoxy-4-androstene-3,17-dione

gift from Dr. A. Brodie, Worcester  
Foundation, Shrewsbury, MS.

aminogluthethimide phosphate

gift from CIBA Pharmaceutical Co.,  
Summit, NJ

## 4.4 Statistical Analyses

Parametric statistical tests were used when data showed no heterogeneity of variance or when any heterogeneity present was removed by logarithmic transformation of the data. For all other statistical analyses nonparametric tests were used. Details of specific tests are given in the individual chapters.

## CHAPTER 5

### PREPARATION AND CHARACTERIZATION OF A SPECIFIC PMSG ANTISERUM

#### 5.1 Introduction

Although recent work has suggested that the half-life of PMSG in rats may be as short as  $\sim 6$  h (Aggarwal and Papkoff, 1981), it has generally been assumed to have a longer half-life of  $\sim 26$  h (Parlow and Ward, 1961). It has been suggested that this long half-life may be the cause of many of the undesirable side-effects associated with the use of PMSG (Gosling et al., 1979).

Desialylation of the PMSG molecule has been reported to decrease its half-life (Jöchle and Lamond, 1980) and its biological activity (Yang and Papkoff, 1973) but most efforts to reduce its time in the circulation have involved the use of specific antisera.

Several authors have used PMSG antiserum in attempts to control superovulation in cattle (Dhondt et al., 1978; Bouters et al., 1980; Kummer et al., 1980; Saumande and Chupin, 1981). Relative to superovulation with PMSG alone, these authors have variously reported, after injection of antiserum, a 50% decrease in the length of oestrus (Kummer et al., 1980), an increased number of ovulations and increased fertilization rate (Dhondt et al., 1978), increased embryo recovery rates (Kummer et al., 1980), an increased percentage of viable embryos (Kummer et al., 1980; Saumande and Chupin,

1981) and a decrease in the number of large unruptured follicles (Dhondt et al., 1978; Kummer et al., 1980; Saumande and Chupin, 1981). Thus in general antiserum appeared to improve the results of superovulation. Looking at hormone levels Kummer et al. (1980) found no difference between serum progesterone concentrations in antiserum-treated and untreated superovulated cattle on day 1-7 after oestrus, but the postovulatory rise in oestradiol concentrations, seen in untreated animals, was prevented by the use of antiserum.

Studies on the mechanism of action of PMSG have also been undertaken using an antiserum to neutralize the hormone. Thus Sasamoto (1969) determined that PMSG need only be present in the circulation for 2 h to exert its luteinizing hormone-like effect and to induce ovulation in immature mice, given a priming dose of PMSG 54-56 h earlier. Sashida and Johnson (1975) showed that maintenance of both the rapid increase in glycolysis and the slower increase in oestradiol secretion, which occur 1 and 20 h respectively after PMSG injection in the rat, required the continued presence of the hormone. Greenwald (1963, 1973) has used a PMSG antiserum to show that, in hamsters superovulated with PMSG the continued presence of the hormone was necessary for maturation of the reserve follicles, which produce most of the oestrogen. In addition, the developing follicles which had been maintained by endogenous gonadotrophin became dependent on PMSG until ovulation occurred. Similar results were found by Sasamoto and Kennan (1972) in hypophysectomized rats. These authors concluded that PMSG was needed continuously in the circulation to maintain follicles until at least 3 h before administration of ovulating

hormone'. However, in intact immature rats, PMSG seems to be necessary only during the initial 36 h, after which time it may be neutralized and endogenous gonadotrophins are responsible for the further development and maintenance of the follicles in a state capable of ovulation (Sasamoto and Kennan, 1973). Chang and Ryan (1976) have suggested that the independence from exogenous gonadotrophin may be related to the appearance of microvilli on the granulosa cells at this time.

This chapter describes the preparation and characterization of a PMSG antiserum, which is used in subsequent chapters to limit the period of action of PMSG.

## 5.2 Methods and Results

### 5.2.1 Preparation and Titration

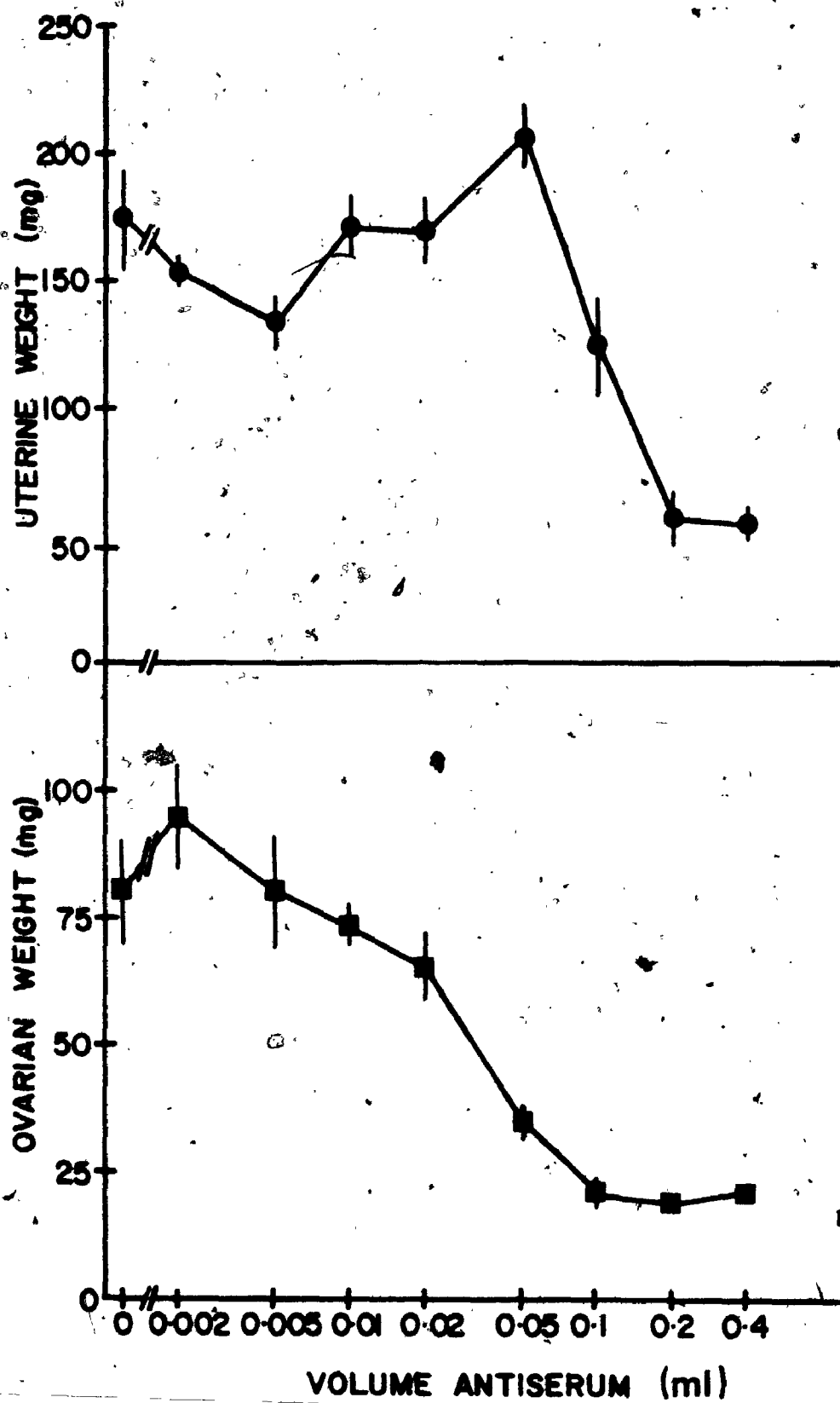
Antiserum (a/s) was produced by immunization of an adult goat with PMSG in Freund's complete adjuvant. The a/s was titrated by measurement of its ability to inhibit increases in ovarian and uterine wet weights, produced by injection of PMSG in immature rats. Different volumes of a/s were injected intraperitoneally 30 min prior to injection of 40 i.u. PMSG at 0800-0830 h. Control animals received normal goat serum (NGS). Rats were sacrificed 48 h later and ovarian and uterine weights were recorded.

Figure 1 shows the results of injecting increasing volumes of a/s on ovarian and uterine weights. Administration of

FIGURE 1

Effect of volume of antiserum on the increase in ovarian and uterine weights induced by 40 i.u. PMSG. All volumes were adjusted to 0.4ml with saline prior to injection. (Mean  $\pm$  S.E.M., n = 5). Standard errors not shown fall within the points as drawn. ●, ■ represents PMSG + a/s treated rats; ○, □ represents rats receiving neither PMSG nor a/s.





40 i.u. PMSG resulted in a 4- to 5-fold increase in ovarian weight which was completely blocked by 0.1 ml of a/s, but 0.2 ml of a/s was required to inhibit the effect on uterine weight. In subsequent experiments an arbitrary dose of 0.2 ml a/s was injected. Injection of a/s alone had no effect on either weight.

### 5.2.2 Cross Reactivity

The cross reactivity of rat LH with PMSG a/s was assessed by measuring the binding of iodinated rat LH to PMSG a/s diluted over the range 1:20 to 1:10,000. A 1:20 dilution of a/s was chosen as the most concentrated since this is a higher concentration than would be expected even if the a/s had been injected i.v. (assuming blood volume is approximately 8% of body weight) and not i.p. as was the procedure in all experiments with a/s. Rat LH was iodinated with  $^{125}\text{I}$  using the chloramine-T method (Greenwood *et al.*, 1963) and 100  $\mu\text{l}$  of the iodinated LH was incubated for 12 h at 37°C with 100  $\mu\text{l}$  of diluted a/s. Bound and free hormones were separated with 10% polyethylene glycol. To check that assay conditions were appropriate an a/s to rat LH (gift from C.S. Sheela Rani) was included in the study.

After correction for binding by NGS and at a dilution of 1 in 20 PMSG a/s bound 14.7% of iodinated LH compared to 66.8% binding by the same dilution of LH a/s (Figure 2). At 1 in 1,000 dilution PMSG a/s bound no iodinated LH compared to 59.1% binding by LH a/s.

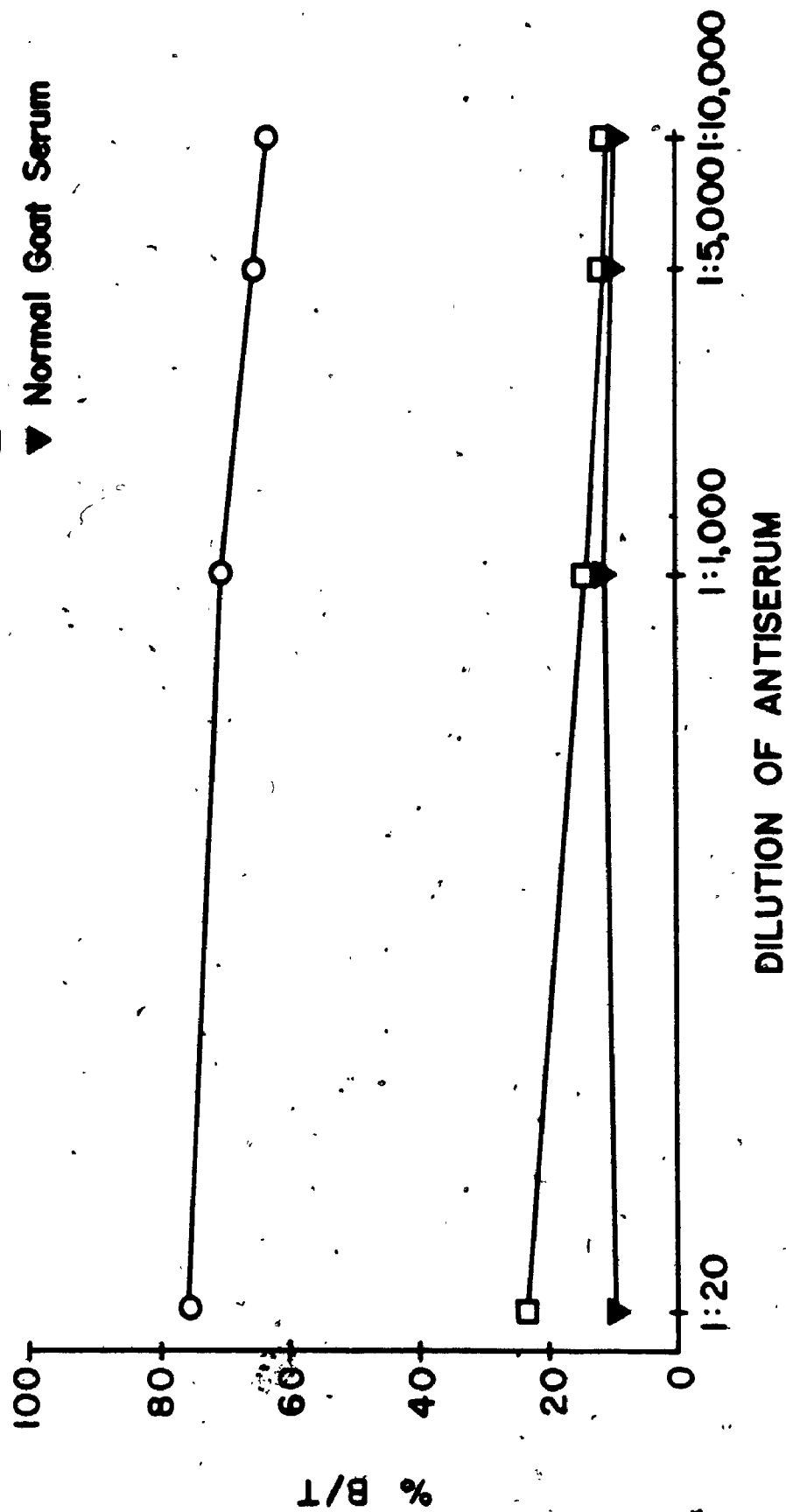
### 5.2.3 Time of Administration

Immature rats were injected with 40 i.u. PMSG on day -2 and a/s was injected at 1800 h on day -1 or day 0. The rats were not

FIGURE 2

Cross reactivity of PMSG antiserum with rat LH  
(B/T = Bound counts per minute/Total counts per minute).

○ LH a/s  
□ PMSG a/s  
▼ Normal Goat Serum



mated overnight, but on the morning of day 1 they were sacrificed. The oviducts were separated from the uterine horns at the uterotubal junction and under a stereoscopic dissecting microscope the oviducts were teased out until relatively straight. A sharp 30-gauge needle was inserted into the oviduct through the uterotubal junction, 0.2 ml DBS was flushed through the oviduct and oocytes were collected in a glass dish. The number and appearance of recovered oocytes were recorded. To determine if the injection of PMSG a/s on day -1 was preventing ovulation by inhibiting an endogenous LH surge on day 0, in a later experiment, a further group consisted of rats injected with 40 i.u. PMSG, followed by a/s at 1800 h on day -1; LH (10 µg NIH-LH-B4, i.p.) was injected at 1600 h on day 0 and these rats were also not mated and were sacrificed on the morning of day 1. In each experiment a t test was used to compare the treatments, with respect to mean oocyte recovery.

Following injection of 40 i.u. PMSG on day -2, very few oocytes were recovered on day 1 when a/s was administered on day -1 compared to a/s on day 0 (Table 1). After injection of a/s on day -1 most oocytes were retained within the follicle, since random puncturing of follicles followed by gentle pressure resulted in the release of an oocyte from most follicles. Injection of LH on day 0 did not cause ovulation of these follicles (Table 2), since there was no significant difference in the mean oocyte recovery with or without LH.

Two distinct populations of oocytes were observed, in most rats, following injection of PMSG a/s on either day. One group comprised oocytes surrounded by cumulus cells and the other group comprised oocytes with no surrounding cumulus complex and showing

TABLE 1

Number of Oocytes Recovered on Day 1  
following Superovulation with 40 i.u. PMSG  
and Injection of Antiserum on Day -1 or Day 0 (mean  $\pm$  S.E.M.; n = 5)

Day PMSG a/s Injected	Oocytes Recovered	
	1-Cell	Fragmented
-1	4.8 $\pm$ 2.6	2.8 $\pm$ 1.0
0	25.0 $\pm$ 2.4*	1.8 $\pm$ 1.1

\*significantly different from PMSG a/s on day -1 ( $P < 0.01$ ).

TABLE 2

Number of Oocytes Recovered on Day 1 following Superovulation  
with 40 i.u. PMSG, Injection of PMSG Antiserum on Day -1  
and LH or Vehicle on Day 0 (mean  $\pm$  S.E.M.; n = 6)

<u>LH</u>	<u>Oocytes Recovered</u>	
	<u>1-Cell*</u>	<u>Fragmented*</u>
-	0.8 $\pm$ 0.8	7.5 $\pm$ 2.6
+	6.7 $\pm$ 6.3	10.2 $\pm$ 2.0

\* Within the classes of oocytes the means were not significantly different.

evidence of degenerative changes (fragmented oocytes). Comparing the two times of injection of a/s there was no significant difference in the mean number of fragmented oocytes recovered (Table 1). The number of 1-cell oocytes surrounded by cumulus was however, significantly higher ( $P < 0.001$ ) after injection of a/s on day 0. The number of oocytes in either group was not significantly different after injection of LH.

### 5.3 Discussion

McCormack and Bennin (1970) have shown that cumulus cells remain attached to oocytes for approximately 20 h after ovulation. Thus, from the different groups of oocytes recovered on day 1 it may be concluded that a/s injected at 1800 h on day -1 (33-34 h after PMSG) blocked ovulation of most follicles destined to ovulate on the third night after PMSG (recovered as 1-cell oocytes surrounded by cumulus), but had no effect on the ovulation of the second night (recovered as naked and fragmented oocytes on day 1). Sasamoto and Kennan (1973) found that in 26-day-old rats given a/s 36 h after 3 i.u. PMSG there was no significant difference in the percentage of rats ovulating overnight of day 28 (82% vs. 92% in the controls), whereas a/s given 30 h after PMSG blocked ovulation in 54% of rats. In the present experiment a/s given 33-34 h after PMSG did not completely block ovulation in any animal, but the mean number of ovulations was significantly reduced and administration of LH on the afternoon of day 0 could



not reverse this effect, suggesting that PMSG a/s was not just blocking the endogenous LH surge. However, the LH receptors present in the ovary after stimulation with PMSG (Bortolussi et al, 1977) could be blocked by the binding of PMSG a/s to ovarian bound PMSG. Hence the follicle would be unable to respond to the endogenous LH surge. From their data, Sasamoto and Kennan (1973) suggested that endogenous gonadotrophins were able to maintain follicles in rats which had 'ovulable' follicles at the time of neutralization of PMSG, but follicles were unable to develop to an 'ovulable' state if supported only by endogenous gonadotrophin. Thus they suggested that PMSG may be needed for initial stimulation of follicular development, but may not always be necessary for further follicular growth, maturation and maintenance. This is in contrast to the situation in the adult hamster, where PMSG stimulated the development of follicles which were dependent on PMSG until ovulation, since administration of a/s on day 4 of the cycle completely blocked ovulation (Greenwald, 1963). The present study suggests that PMSG is needed for follicular development for longer than 33-34 h, but it cannot be determined if, at a later stage, the follicles could be maintained by endogenous gonadotrophin alone. In addition, the situation in the immature superovulated rat may not be exactly comparable to that in the immature rat treated with 3 i.u. PMSG (Sasamoto and Kennan, 1973).

From data on uterine weight changes Sasamoto and Kennan (1973) suggested that oestradiol concentrations after a/s at 36 h are lower than in PMSG-treated controls, but these concentrations could still stimulate the release of LH on the afternoon of the second day after PMSG. In the present study the cross reactivity of PMSG a/s

with LH was relatively low (Figure 2) and the presence of a/s administered on day -1 was therefore unlikely to inhibit the action of the endogenous LH surge on day 0. This is confirmed by the inability of exogenous LH to induce additional ovulations. When a/s was given on day 0 oestradiol concentrations would presumably have remained high until the time of, or just prior to, the endogenous LH surge and no inhibition would have occurred.

Thus an antiserum to PMSG was prepared which could neutralize exogenous PMSG while cross reacting very little with endogenous rat LH. Use of this antiserum is described in subsequent chapters.

## CHAPTER 6

### SUPEROVULATION AND PREGNANCY IN IMMATURE RATS

#### 6.1 Introduction

Nuti et al. (1975) have shown that 8 i.u. PMSG administered to immature rats can induce ovulation and an apparently normal pregnancy. With higher doses of PMSG (up to 16 i.u.) Miller and Armstrong (1981a) observed that the number of fetuses per pregnant rat increased, but implantation was delayed in a proportion of animals, and the proportion of rats which carried their fetuses to day 20 was also decreased. Miller and Armstrong (1981b) also showed that superovulatory doses of 40 i.u. PMSG resulted in infertility, as judged by the presence of degenerate oocytes in the reproductive tract on day 3 and the absence of blastocysts in the uterus on day 5 after ovulation and mating.

If, after treatment with 40 i.u. PMSG, the rat was ovariectomized within 24 h of ovulation and fertilization, and a hormonal replacement regimen known to sensitize the uteri for the decidual cell reaction was given (Kennedy, 1979), the rats frequently became pregnant and apparently normal fetuses were recovered at sacrifice on day 20 (Miller and Armstrong, 1982). These results suggest that it was the failure of the ovary to provide a suitable hormonal

6

environment rather than defects in oocyte maturation, ovulation, fertilization or implantation per se which prevented pregnancy in the PMSG-superovulated rat.

Development of rabbit embryos is inhibited in oestrogen-dominated oviducts (Stone et al., 1977), and mouse embryos cultured in oviductal fluid from oestrogen-dominated donors were less able to develop compared to those cultured in media or fluid from progesterone-dominated oviducts (Cline et al., 1977). Levels of oestrogen in the ovaries and blood of the PMSG-superovulated rat were very high on days 1-3 of pregnancy compared to levels in control rats given 4 i.u. (Miller and Armstrong, 1981b). It therefore seems probable that the high dose of PMSG, because of its prolonged biological half-life (Sasamoto et al., 1972), stimulated the ovary to produce oestrogen in larger than normal amounts and for longer than normal periods, leading to an interference with pregnancy. If the duration of action of PMSG could be shortened pregnancy might then occur in the intact animal. In the present chapter the period of action of PMSG was restricted to ~ 58 h by the use of an antiserum, and the effect on early pregnancy in superovulated animals was determined. The work of Miller and Armstrong (1981a) had indicated that the delayed implantation, observed in approximately 30% of immature rats injected with 16 i.u. PMSG, could be overcome by administration of oestrogen on day 4 (nidatory oestrogen). Nidatory oestrogen was therefore given to a group of superovulated antiserum-treated rats in case delay should prove to be a problem.

The use of gonadotrophic preparations with shorter

half-lives (e.g., pituitary FSH preparations) may overcome some of the endocrine problems associated with the prolonged action of PMSG. However, these preparations have the disadvantage of requiring frequent injections or infusion to maintain biologically active levels. FSH was therefore continuously infused subcutaneously and the resulting superovulation and pregnancy were compared with those resulting from a single injection of PMSG.

## 6.2 Superovulation with PMSG

### 6.2.1 Methods

6.2.1.1 Experimental Design: Immature rats were randomly allocated to 1 of 4 treatment groups. Control animals received 4 i.u. PMSG s.c. and all other rats received a superovulatory dose of 40 i.u. PMSG, at 0830 h on day -2. Superovulated rats were divided into 3 groups, and received either no further injections (SOV), 0.2 ml PMSG a/s at 1800 h on day 0 (SOV a/s) or the same dose of a/s at 1800 h on day 0, followed by an injection of nidatory oestrogen (50 ng oestradiol) on day 4 (SOV a/s  $E_2$ ).

6.2.1.2 Collection of Data: Animals were sacrificed by decapitation at 1030-1230 h on each of days -2 to 5, 7, 8 and 20 of pregnancy and trunk blood was collected. The ovaries were immediately weighed and transferred to chilled ethanol. On days 1-5 the oviducts

were separated from the uterine horns at the uterotubal junction and under a stereoscopic dissecting microscope the oviducts were teased out until relatively straight. A sharp 30-gauge needle was inserted into the oviduct through the uterotubal junction, 0.2 ml DBS was flushed through the oviduct and oocytes and embryos were collected in a glass dish. To facilitate counting on day 1, oocytes were exposed to 0.1% hyaluronidase for 5 min to denude oocytes of cumulus cells. Uteri, on days 1-5, were flushed with 0.5 ml DBS by inserting a sharp 25-gauge needle through the wall at the oviductal end. They were subsequently flushed with a further 0.3 ml DBS from the cervical end. Uteri were then blotted to express any remaining intraluminal fluid and were weighed. Oocytes and embryos recovered were counted and some were examined under a phase contrast microscope.

Implantation sites in the uterus were counted when present on days 7 and 8 and the uteri were flushed to recover any free blastocysts. On day 20 the total number of foetuses, together with the number of live foetuses, was recorded. Total foetal and placental weights were recorded as a measure of prenatal foetal development, and when no live foetuses were recovered the uteri were examined for any evidence of implantation.

On days 2 and 3 of pregnancy 5 additional rats in each of the SOV and SOV a/s-treated groups were sacrificed by cervical dislocation, one ovary was dissected free from each animal, and was ~~fixed~~ in Bouin's fluid for subsequent histological examination.

6.2.1.3 Blood and Tissue Analyses: Serum was separated from the blood on the day of collection and stored at -20°C until

analysed for steroids using specific RIA techniques. Serum oestradiol was extracted with diethyl ether and assayed using [2,4,6,7,16,17-<sup>3</sup>H]-oestradiol-17 $\beta$  (New England Nuclear, 130-170 Ci/mmol) and an antibody raised against oestradiol-17 $\beta$ -6-carboxymethyloxime. While the oestradiol assay has not been completely validated, cross reactivity with other steroids is low (oestrone < 3%, testosterone < 0.02%, progesterone < 0.01%). Progesterone was extracted from serum with redistilled petroleum ether (40-60°C) and assayed using an iodinated tracer as described previously (Leung and Armstrong, 1979). In both assays ether was dried down in the standard curve tubes and PBS blanks in all cases were zero. Recoveries were estimated by addition of known amounts of oestradiol or progesterone to buffer or charcoal-treated, steroid-free serum. The regression of recovered on added hormone was described by  $Y = (1.04 \pm 0.03) X + 6.43$  and  $Y = (0.87 \pm 0.04) X + 0.62$  for oestradiol and progesterone respectively, where Y = recovered hormone and X = added hormone. The coefficients of both regressions were highly significantly ( $P < 0.001$ ) different from zero. The mean within assay coefficient of variation for oestradiol for two serum pools was 7.83%, when measured 5 times in the same assay, and the mean between assay coefficient of variation was 8.30%, when measured in 3 separate assays. The corresponding values for the progesterone assay were 12.68% and 14.08% for the mean within assay and between assay coefficients of variation, respectively.

Ovaries were homogenized in chilled ethanol immediately after collection and the homogenate was stored at -20°C until analyzed. The homogenate was centrifuged and aliquots of the ethanolic supernatant

were evaporated to dryness and redissolved in buffer. RIA was carried out utilizing the same antibodies and assay systems described for the serum analyses. Reference pools were included in both assays and the mean within assay coefficients of variation were 6.60% and 12.58% for oestradiol and progesterone respectively. Between assay coefficients of variation were 13.02% and 14.10% for oestradiol and progesterone respectively.

The percentage cross reactivity of PMSG with LH a/s was measured at 50% maximum binding under assay conditions described previously (Weick, 1977).

6.2.1.4 Histological Examination: Tissue for histological examination was preserved in Bouin's fluid and embedded in paraffin wax. Serial sections of the specimens were cut at 25  $\mu$ m and stained with haematoxylin and eosin. The central 10 sections of each ovary were projected overhead using a Bausch and Lomb Tri-simplex overhead projector and the diameter of all follicles, where the oocyte was visible, was measured. A stage micrometer was used for calibration. Where follicles were irregularly shaped the largest diameter was measured. After arbitrary division into classes the measurements were plotted on a histogram.

6.2.1.5 Statistical Analyses: For tissue weights and concentrations of ovarian and serum steroids the significance of differences between control, SOV and SOV a/s rats were tested by the Friedman method of nonparametric analysis of variance (Siegel, 1956), for the period days 1-5. SOV and SOV a/s-treated animals were compared and a second comparison was made between SOV a/s and control rats, to test the hypothesis that treatment with a/s returns the animals to a more physiological state, with respect to tissue weights, hormone concentrations and embryo recovery.



On days 7, 8 and 20 the effects of treatments were compared using the Kruskal Wallis One Way Analysis of Variance (Siegel, 1956). For weights of implantation sites, fetuses and placentae the analyses were based on between animal variation. Differences between pairs of treatments were compared using the Wilcoxon Critical Range method (Colquhoun, 1971) when samples were of equal size. For samples of unequal size pairs of treatments were compared using a Mann-Whitney U test (Siegel, 1956).

Within treatments differences between days 5, 8 and 20 were compared using one way Analysis of Variance and Duncan's New Multiple Range test (Steel and Torrie, 1960).

On day 20 the correlations of number of fetuses and foetal and placental weights were estimated using the Spearman Rank Correlation Coefficient (Colquhoun, 1971). Following histological preparation, the mean number of follicles in each size class from ovaries of rats on days 2 and 3 of pregnancy following superovulation with or without treatment with a/s, were compared using a t-test.

### 6.2.2 Results

#### 6.2.2.1 Effect of a/s on Oocyte, Embryo and Placental

Recovery: Oocyte recovery rates indicate that in control animals receiving 4 i.u. PMSG ovulation occurred between days 0 and 1, whereas in SOV rats treated with 40 i.u. PMSG ovulation occurred between days -1 and 0 and again between days 0 and 1, regardless of whether or not a/s was administered (Table 3). Table 3 also shows that treatment with a/s resulted in a higher number of embryos being recovered on

TABLE 3

Number of Oocytes and/or Embryos Recovered on Days 1-5 of Pregnancy  
 in Control, SOV and SOV a/s-Treated Rats  
 (mean  $\pm$  S.E.M.; n = 10 rats per treatment per day)

<u>Day of Pregnancy</u>	<u>Treatment</u>		
	<u>Control</u>	<u>SOV</u>	<u>SOV a/s</u>
0	0.0	7.6 $\pm$ 1.3	*
1	9.9 $\pm$ 0.9	21.8 $\pm$ 4.0	23.8 $\pm$ 4.8
2	8.2 $\pm$ 1.1	12.4 $\pm$ 4.4	17.5 $\pm$ 5.7
3	6.0 $\pm$ 0.9	9.7 $\pm$ 4.1	13.9 $\pm$ 2.6
4	6.6 $\pm$ 1.0	1.1 $\pm$ 0.7	9.7 $\pm$ 2.9
5	8.2 $\pm$ 0.5	2.4 $\pm$ 1.9	10.5 $\pm$ 2.4

\* No rats in this group were killed prior to day 1, since a/s was not injected until 1800 h on day 0.

days 4 and 5 compared to SOV untreated animals. Control animals had a mean of 7.9 oocytes or embryos over the first 5 days of pregnancy, whereas injection of 40 i.u. PMSG resulted in an overall mean of 22.8 oocytes or embryos on day 1 which dropped by day 5 to 2.4 in the SOV group compared to 10.5 in the SOV a/s rats. In both SOV and SOV a/s animals the greatest drop in numbers occurred between days 1 and 3. Over the period days 1-5 the differences in embryo recovery rates between SOV untreated and SOV a/s rats and between SOV a/s and control rats were statistically significant ( $P = 0.025$ ) in both cases. In control animals embryos were recovered from 100% of animals on day 5 and embryos were found only in the uterus, whereas in SOV animals in the absence of a/s embryos were recovered from only 30% of animals on day 5. Under the phase contrast microscope these embryos usually appeared grossly abnormal, displaying irregularly sized and shaped cells with granular cytoplasm. In contrast, 100% of animals treated with a/s had embryos on day 5, most of which appeared normal under the phase contrast microscope. In the majority of these animals embryos were found only in the uterus, whereas in SOV animals which did not receive a/s embryos were never recovered predominantly from the uterus (Figure 3).

Figure 4 and Table 4 show that implantation sites were found in 85% of control rats on day 7 and 100% of control rats on day 8, with means, for those pregnant, of 8.0 and 9.6 implantation sites on days 7 and 8 respectively (ranges 1-13 and 2-13 respectively). In SOV animals there was no evidence of implantation on either day. In SOV a/s rats only 40% of animals had implantation sites on day 7; these were usually very small and it was not possible to obtain accurate counts.

### FIGURE 3

Percentage of rats with oocytes and/or embryos on days 0 to 5 of pregnancy and location of these oocytes/embryos. In each treatment on each day there were 10 rats (— denotes 0%). Animals were injected with 4 i.u. PMSG (control) or 40 i.u. (SOV) on day -2; 50% of SOV rats received PMSG a/s on day 0.

2



1.0



1.1



1.25



1.4



1.6



1.8



2.0



2.2



2.5



2.8



3.2



3.6



4.0



PERCENTAGE OF RATS WITH OOCYTES AND/OR EMBRYOS

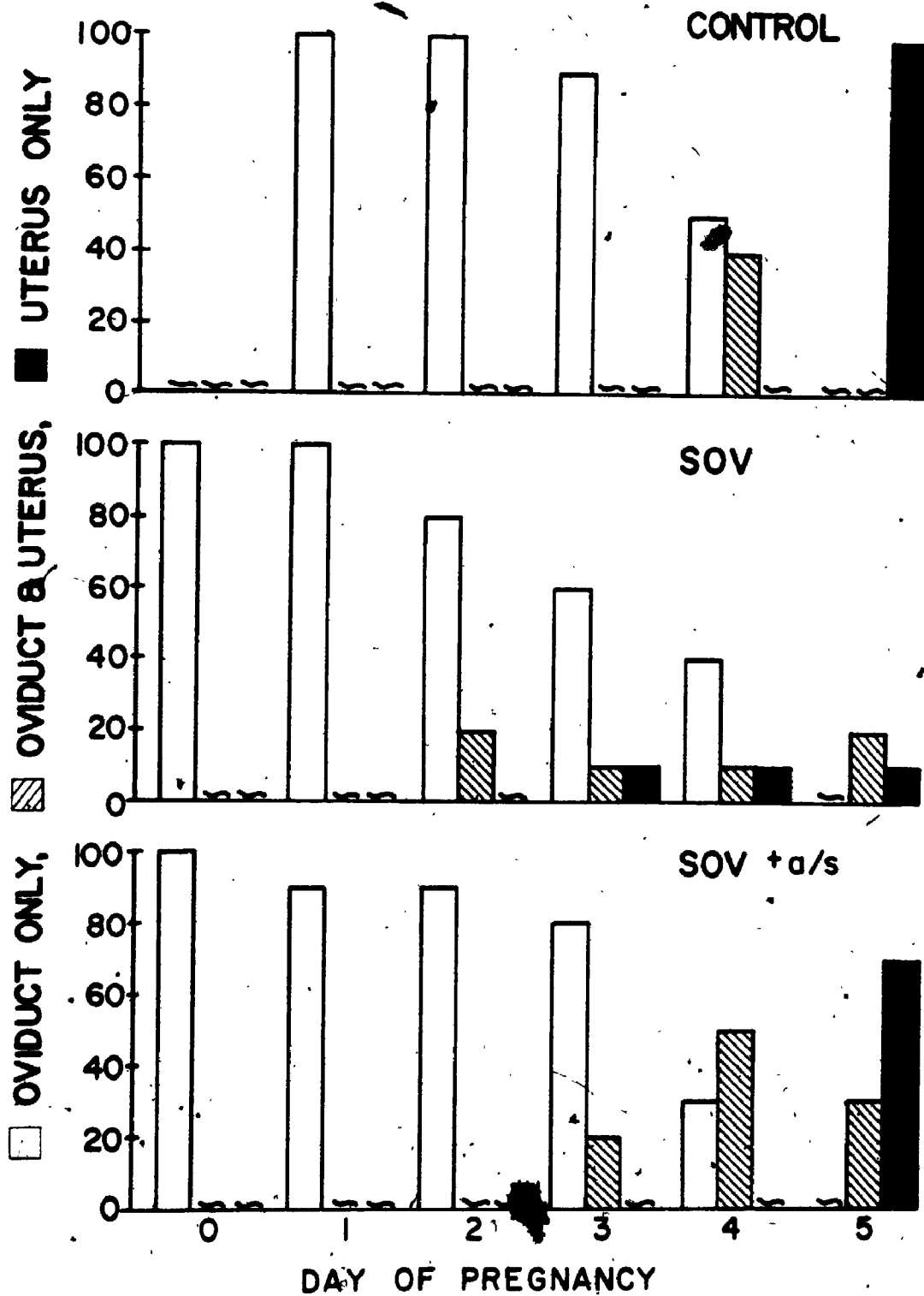
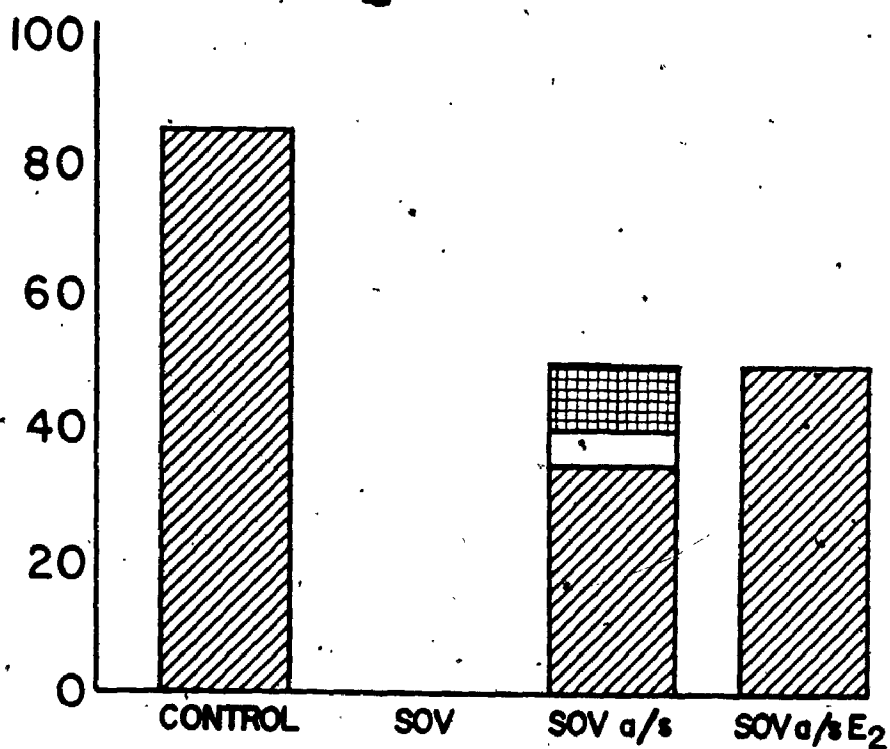


FIGURE 4

Percentage of rats with implantation sites and/or free blastocysts on days 7 and 8 of pregnancy. Animals were injected with 4 i.u. PMSG (control) or 40 i.u. Rats injected with 40 i.u. were divided into 3 groups which subsequently received no further treatment (SOV), PMSG antiserum (SOV a/s) or PMSG antiserum and nidatory oestrogen (SOV a/s  $E_2$ ). (n = 20 rats per treatment on each day).

PERCENTAGE OF RATS

DAY 7



DAY 8

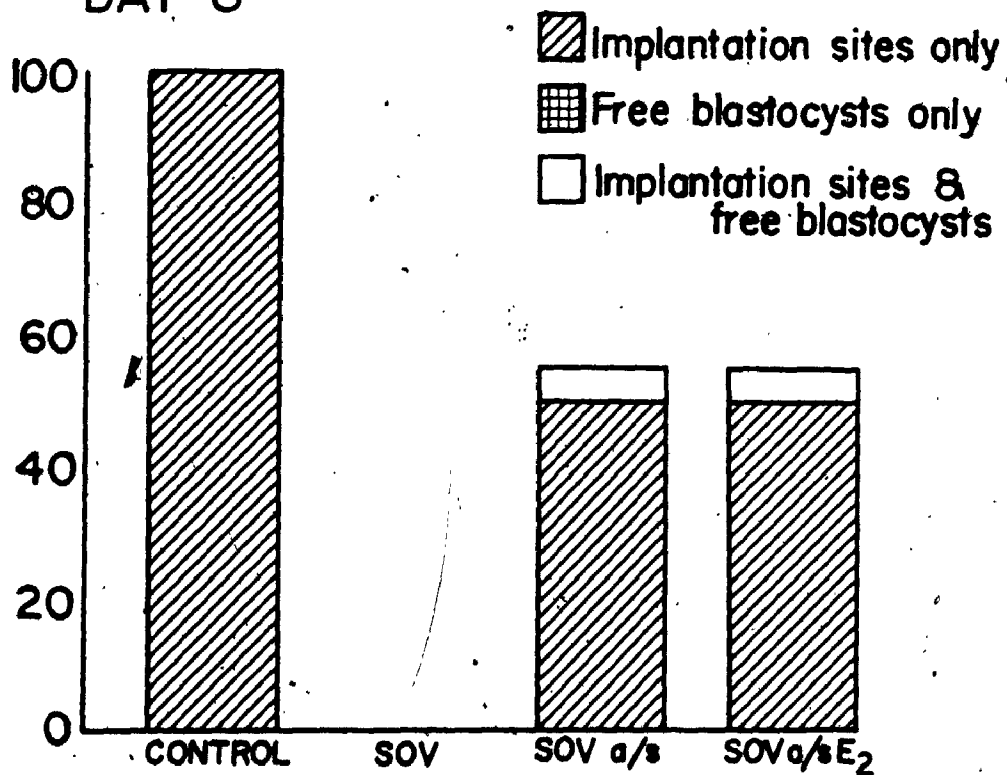




TABLE 4

Proportion of Rats Pregnant and Implantation Site Numbers and Weight on Day 8 after Ovulation (mean  $\pm$  S.E.M.; for details of treatments see text)

	Treatment			
	Control	SOV	SOV a/s	SOV a/s E <sub>2</sub>
Proportion of rats with implantation sites	20/20	0/20	10/20	10/20
Number of implantation sites	9.6 $\pm$ 0.6 <sup>a</sup>	-	9.6 $\pm$ 3.4 <sup>a</sup>	13.4 $\pm$ 2.7 <sup>a</sup>
Implantation site weight (mg)	17.6 $\pm$ 1.0 <sup>a</sup>	-	13.6 $\pm$ 1.1 <sup>b</sup>	20.5 $\pm$ 1.1 <sup>a</sup>

<sup>a,b</sup> within each variable, means with the same superscript are not significantly different.

A mean of 13.5 blastocysts, all zona-free, were recovered from a further 10% of animals and a single blastocyst was recovered from one animal in which there was evidence of implantation. By day 8 50% of animals showed evidence of implantation with a mean of 9.6 sites per pregnant animal (range 1-39). Similar results were observed in SOV a/s  $E_2$  rats, with 50% of animals having implantation sites on both days, and on day 8 a mean of 13.4 implantation sites (range 1-26) was observed. Comparing control, SOV a/s and SOV a/s  $E_2$  rats which had implantation sites on day 8 there was no significant difference in the number of sites observed; nor was there any significant difference between the numbers of blastocysts on day 5 and implantation sites on day 8. There was, however, a significant difference ( $P < 0.01$ ) in the mean implantation site weight between treatments (Table 4); values for SOV a/s rats were significantly lower ( $P < 0.01$ ) than those for control or SOV a/s  $E_2$  rats.

The percentage of animals pregnant on day 20 is shown in Figure 5 and Table 5. In the control group of rats 80% were pregnant with a mean, for pregnant animals, of 7.8 live fetuses (range 1-12), which is significantly lower ( $P < 0.05$ ) than the number of implantation sites on day 8. In control rats which were not pregnant there was no evidence of implantation, suggesting that the pregnancy loss occurred either before implantation, or very shortly thereafter. SOV rats were never found to be pregnant on day 20 whereas 29% of SOV a/s rats had live fetuses with a mean of 11.6 fetuses (range 2-28). A further 6% of these rats had resorbed fetuses or evidence that implantation had occurred. In SOV a/s  $E_2$  rats 29% were pregnant and

FIGURE 5

Percentage of rats pregnant on day 20. For details of treatments see

Figure 4. (Numbers in brackets represent the number of rats in each group).

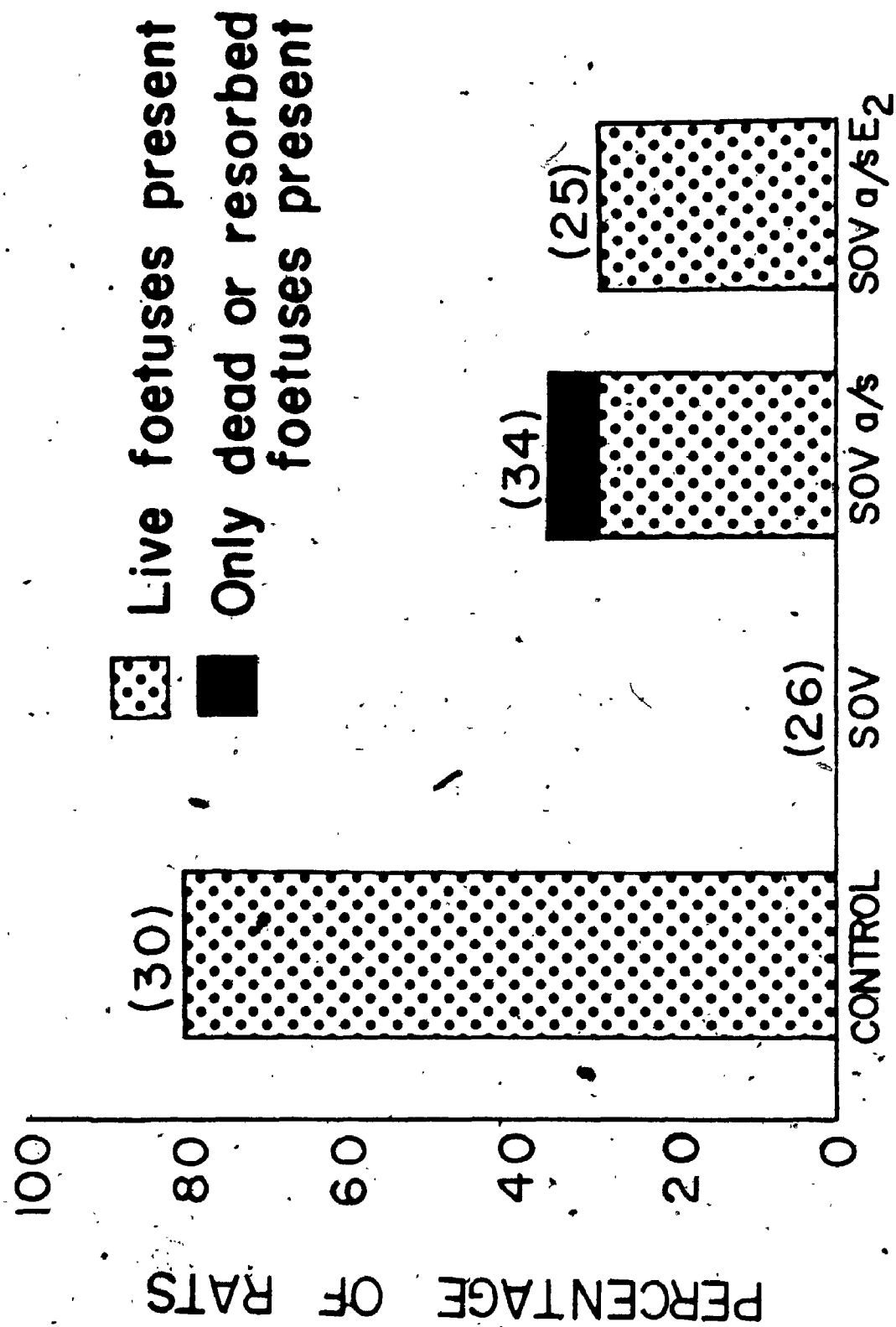


TABLE 5

Recovery of Foetuses and Foetal and Placental Weights  
on Day 20 of Pregnancy (mean  $\pm$  S.E.M.;  
for details of treatments see text)

	Treatment		
	Control	SOV a/s	SOV a/s E <sub>2</sub>
Proportion of rats pregnant	24/30	10/34	7/25
Number of foetuses	7.8 $\pm$ 0.5 <sup>a</sup>	11.6 $\pm$ 2.7 <sup>a</sup>	10.1 $\pm$ 2.6 <sup>a</sup>
Foetal weight* (g)	1.8 $\pm$ 0.1 <sup>a</sup>	1.1 $\pm$ 0.1 <sup>b</sup>	1.8 $\pm$ 0.1 <sup>a</sup>
* Placental weight* (mg)	473 $\pm$ 20 <sup>a</sup>	307 $\pm$ 25 <sup>b</sup>	563 $\pm$ 144 <sup>a</sup>

\* live foetuses only.

<sup>a,b</sup> within each variable, means with the same superscript are not significantly different.

live fetuses were recovered from all animals, with a mean of 10.1 (range 7-18). The number of live fetuses in both superovulated groups was not significantly different from the number of implantation sites on day 8. Several animals in both groups had both live and dead fetuses. Most of the implanted fetuses classified as nonviable on day 20 had apparently died early in pregnancy, although an occasional well-developed dead fetus was seen. There was no significant difference in the number of fetuses recovered from each treatment, but analysis of variance showed that there was a significant difference ( $P < 0.001$ ) between treatments with respect to both foetal and placental weights (Table 5). SOV a/s rats had significantly lower foetal and placental weights than either control ( $P < 0.001$  and  $P < 0.002$ , respectively), or SOV a/s  $E_2$  rats ( $P < 0.001$  and  $P < 0.005$ , respectively).

The correlations of numbers of fetuses and foetal and placental weights are given in Table 6. Significant correlations were generally not observed in the superovulated rats, possibly due to the lower number of rats pregnant. In control animals both foetal and placental weights were negatively correlated with the number of fetuses ( $r = -0.399$ ,  $P < 0.05$  and  $r = -0.776$ ,  $P < 0.01$ , respectively). Foetal and placental weights in control animals were positively correlated ( $r = 0.413$ ,  $P < 0.05$ ).

#### 6.2.2.3 Effect of a/s on Ovarian and Uterine Weights:

In control animals an increase in ovarian weight occurred between days -2 and 1 after which there was no increase over the period days 1-5. Treatment with 40 i.u. PMSG resulted in a much larger

TABLE 6

Correlations of Number of Foetuses and Mean Foetal and Placental Weights on Day 20 of Pregnancy  
(for details of treatment see text)

	Treatment							
	Control (df = 18)		SOV a/s (df = 5)		SOV a/s E <sub>2</sub> (df = 5)		Combined Data (df = 32)	
	r	P	r	P	r	P	r	P
Number of foetuses vs. foetal weight	-0.399	< 0.05	-0.250	NS	-0.423	NS	-0.306	< 0.05
Number of foetuses vs. placental weight	-0.776	< 0.01	-0.536	NS	-0.679	NS	-0.664	< 0.01
Foetal weight vs. placental weight	0.413	< 0.05	0.857	< 0.01	0.679	NS	0.691	< 0.01

increase in ovarian weight, but again after day 1 there was very little change up until day 5; the weight remained around 160 mg in SOV animals and around 120 mg in SOV a/s rats (Figure 6). Uterine weights in all animals increased nearly 4-fold between days -2 and 0 to reach 140-150 mg (Figure 6). Following this, in control animals there was a small decline until day 3 followed by a small increase until day 5. Uterine weights of SOV animals continued to increase after day 0 reaching a peak of 224 mg on day 2. The weight then tended to decline and was reduced to 180 mg by day 5. SOV a/s rats showed a slight increase between days 0 and 1 after which the pattern was similar to control rats, but approximately 20 mg higher on all days. Over the period days 1-5, differences in both ovarian and uterine weights between SOV and SOV a/s rats and between SOV a/s and control rats were statistically significant ( $P = 0.025$ ) in all cases.

Ovarian weights on day 8 were not significantly different to those on day 5, whereas they were significantly higher ( $P < 0.01$ ) for all treatments on day 20 compared to day 8. On both days 8 and 20 ovarian weight was significantly lower in control animals ( $P < 0.01$  and  $P < 0.001$ , respectively) than in the rats receiving the super-ovulatory treatments (Table 7).

#### 6.2.2.3 Effect of a/s on Steroid Hormone Levels:

Between days 1 and 5 ovarian progesterone content rose continuously in all groups (Figure 7). Initially, on day -2 total ovarian progesterone content was 2 ng, but by day 5 levels of 600 ng, 3,000 ng and 2,300 ng were measured in control, SOV and SOV a/s rats respectively. Over the period days 1-5 the difference between control and SOV a/s animals was statistically significant ( $P = 0.025$ ),



FIGURE 6

Ovarian and uterine weights in control, SOV and SOV a/s treated rats. PMSG was injected on day -2 and a/s on day 0. Results are mean  $\pm$  S.E.M. for 10 rats per treatment per day. Standard errors not shown fall within the points as drawn.

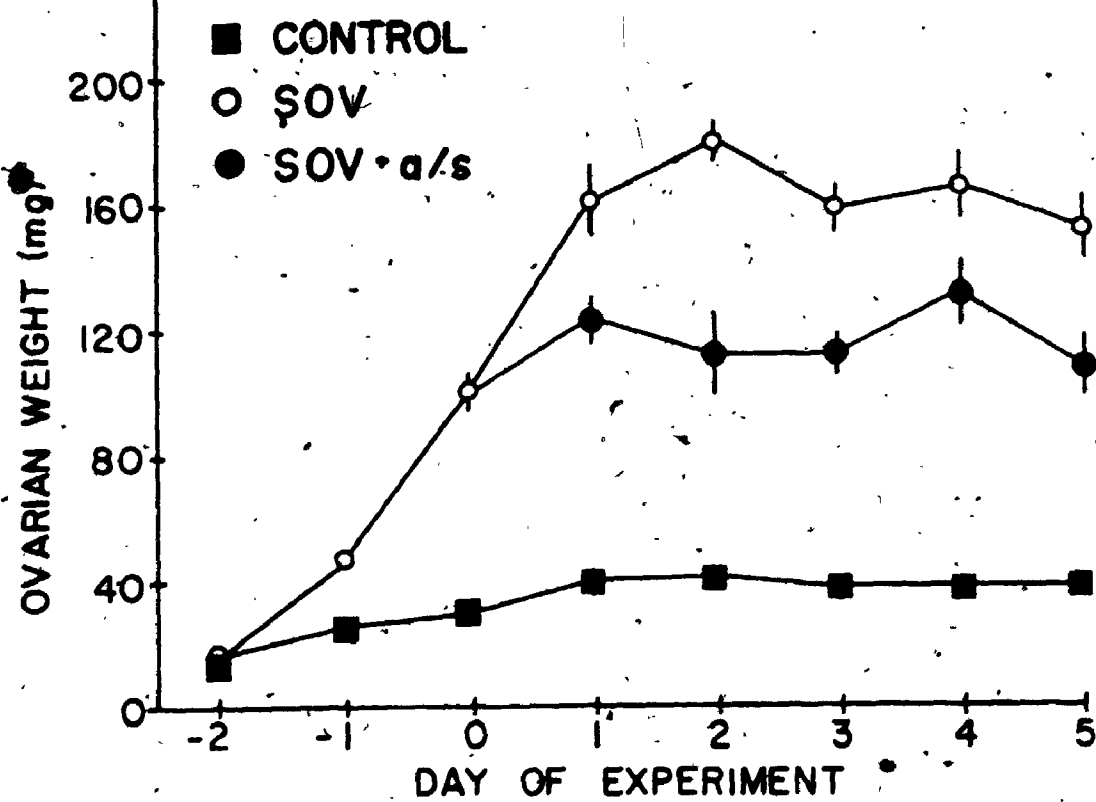
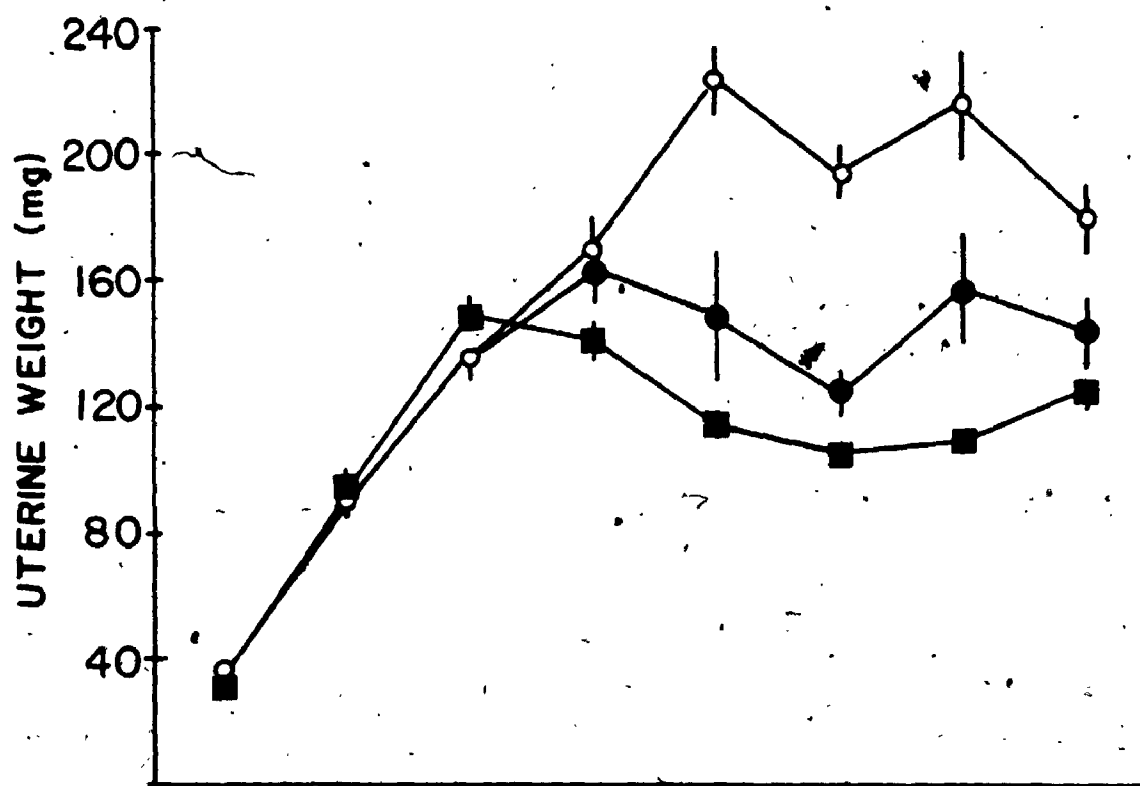


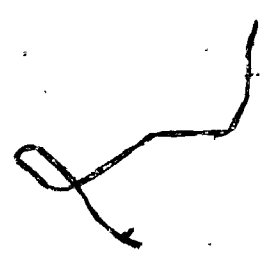
TABLE 7

Ovarian Weights on Days 8 and 20 of Pregnancy (mg; mean  $\pm$  S.E.M.;  
for details of treatments see text)

Day	Treatment		
	Control	SOV a/s	SOV a/s-E <sub>2</sub>
8	39.7 $\pm$ 1.0 <sup>a</sup>	109.2 $\pm$ 5.6 <sup>b</sup>	120.4 $\pm$ 4.4 <sup>b</sup>
20*	71.2 $\pm$ 2.2 <sup>a</sup>	294.8 $\pm$ 198.0 <sup>b</sup>	273.6 $\pm$ 26.9 <sup>b</sup>

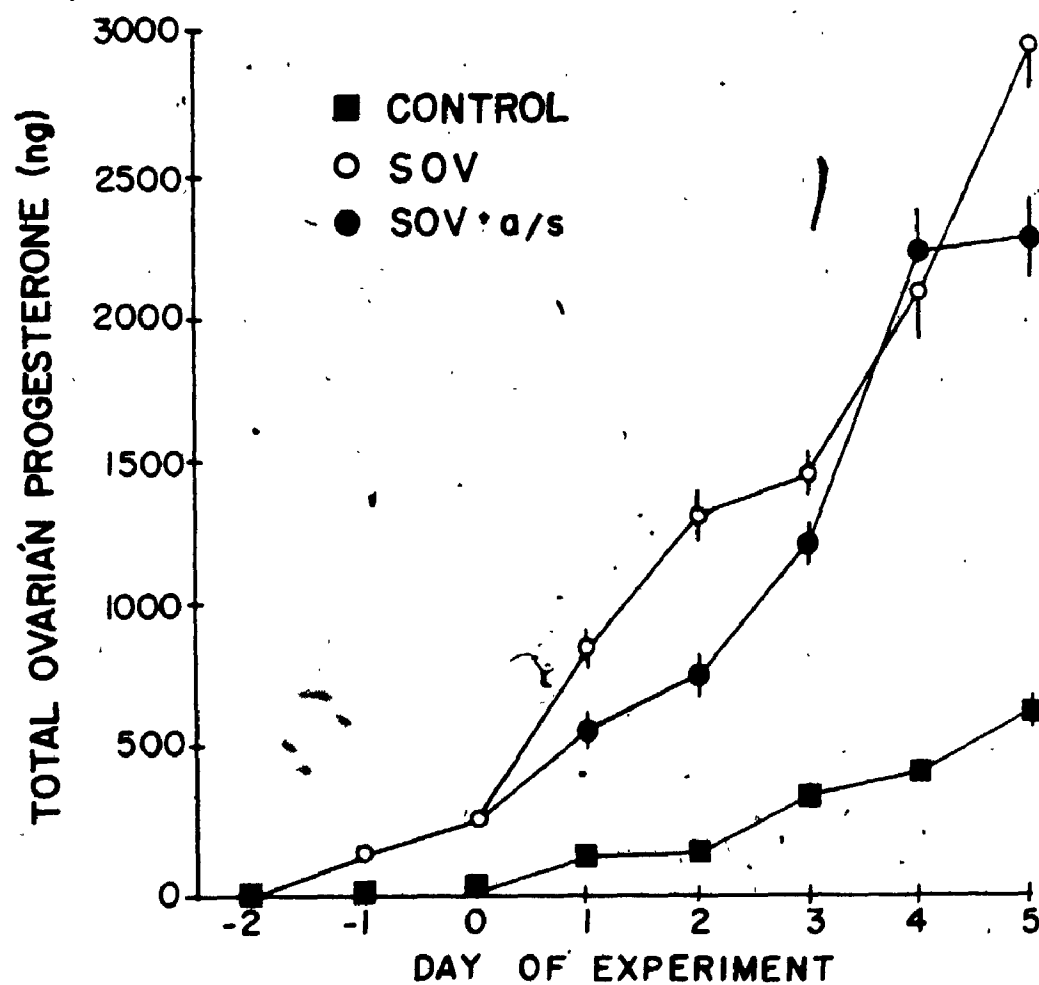
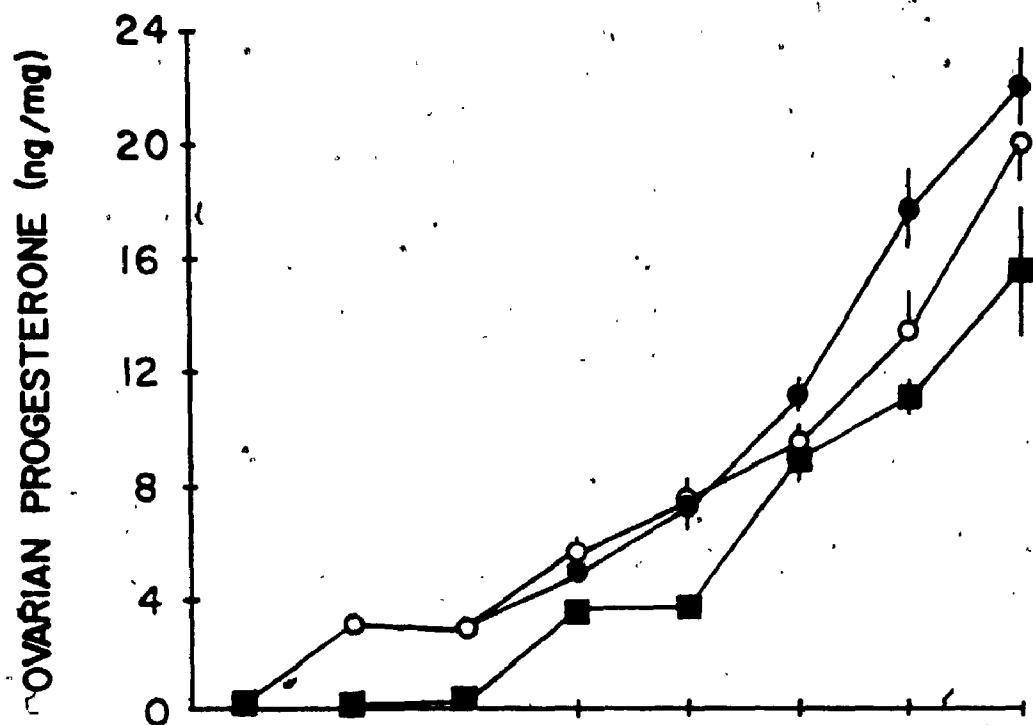
\*with at least one live foetus.

<sup>a, b</sup> within each day, means with the same superscript are not significantly different.



# FIGURE 7

Ovarian progesterone content and concentrations in control, SOV and SOV a/s treated rats. PMSG was injected on day -2 and a/s on day 0. Results are mean  $\pm$  S.E.M. for 10 rats per treatment per day. Standard errors not shown fall within the points as drawn.

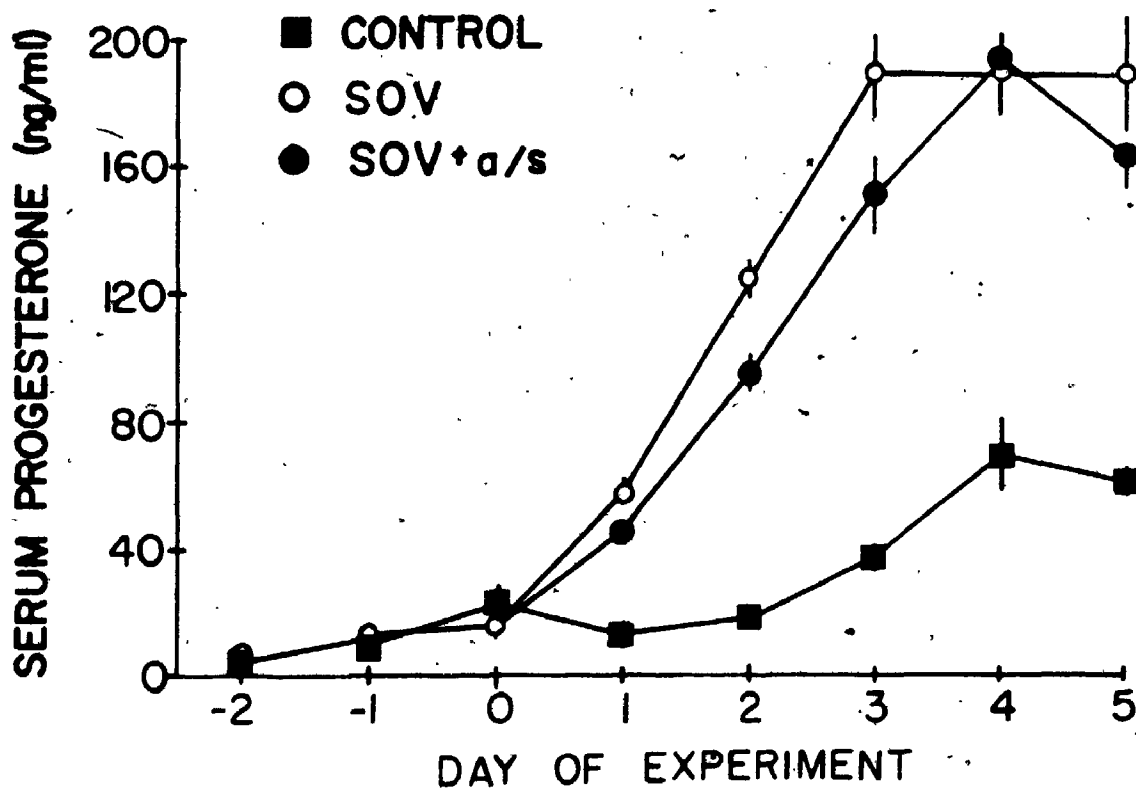
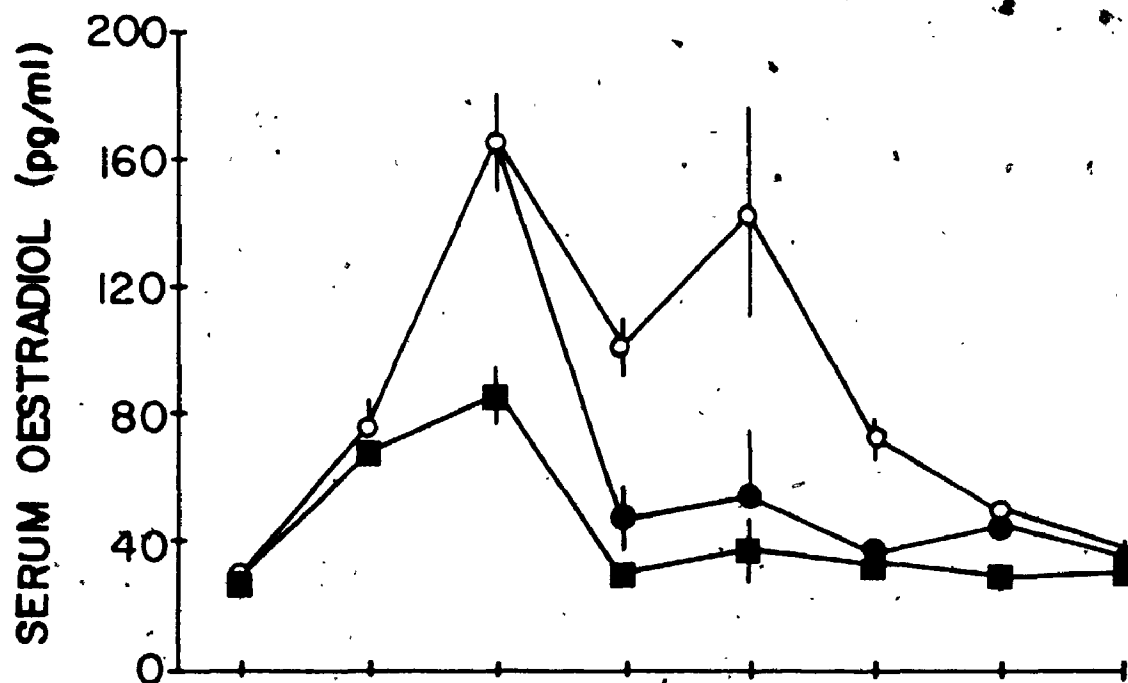


whereas the difference between SOV and SOV a/s rats was not significant. When expressed as ng/mg wet tissue (Figure 7) values were more similar in all treatments, but the difference between SOV a/s and control rats was still statistically significant ( $P = 0.025$ ), SOV a/s rats having the highest concentrations of all three groups. Over the same period a similar pattern was seen in serum progesterone concentrations (Figure 8). Injection of PMSG at either concentration resulted in a 4-fold increase in serum progesterone levels, to reach approximately 20 ng/ml by day 0. Values in control animals remained steady from day 0 to day 2 and then rose to approximately 70 ng/ml by day 4. In SOV and SOV a/s-treated rats levels continued to rise after day 0 reaching approximately 190 ng/ml in SOV animals by day 3 and in SOV a/s rats by day 4. These levels were maintained until day 5. The difference between SOV a/s and control animals was again significant ( $P = 0.025$ ), whereas that between SOV and SOV a/s rats was not.

Total ovarian oestradiol content rose from 0.09 ng on day -2 to 3.2 ng in control rats and 7.9 ng in all SOV rats on day 0 (Figure 9). Following ovulation, in control rats levels fell to 0.14 ng within 12 h and remained low throughout the next 5 days. Injection of a/s caused a similar sharp drop and levels were not statistically different from control rats for the remainder of the experimental period. In the absence of a/s levels remained elevated until day 3, showing a small but definite drop on day 1 followed by a second peak of 8.9 ng on day 2. The difference between SOV untreated and SOV a/s rats was statistically significant ( $P = 0.025$ ). When oestradiol content was expressed as ng/mg wet tissue (Figure 9) there was again no statistically significant difference between control and SOV a/s rats, whereas the

# FIGURE 8

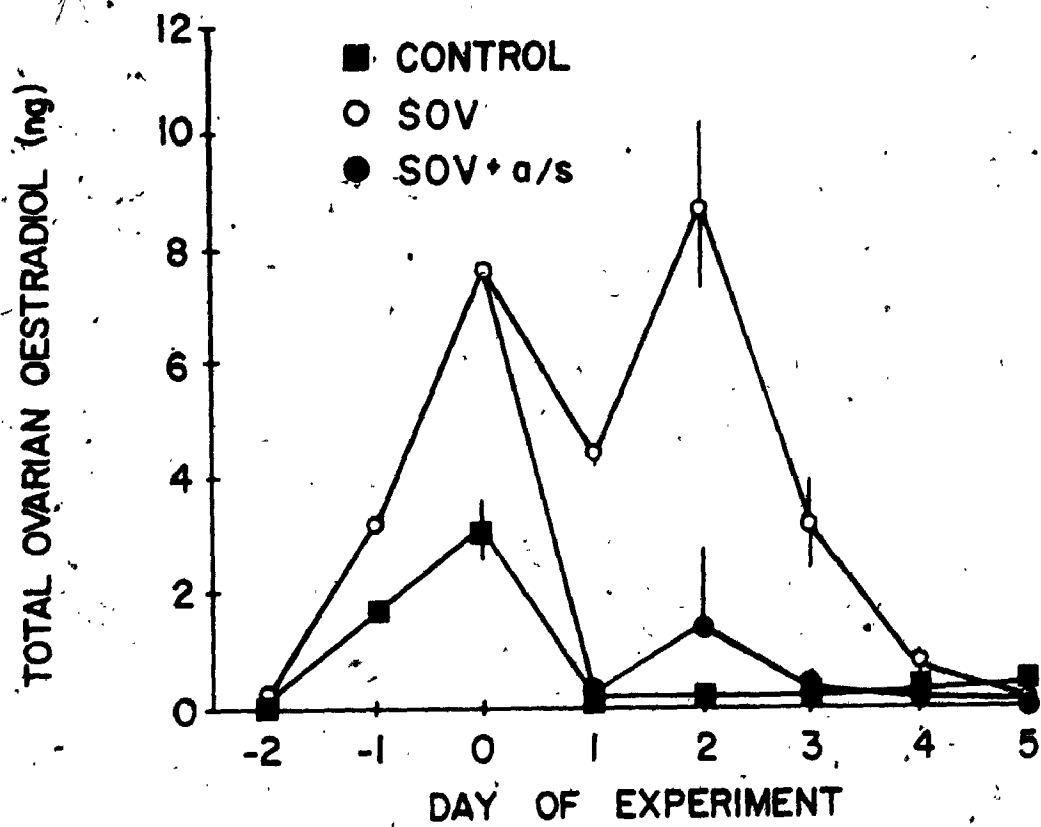
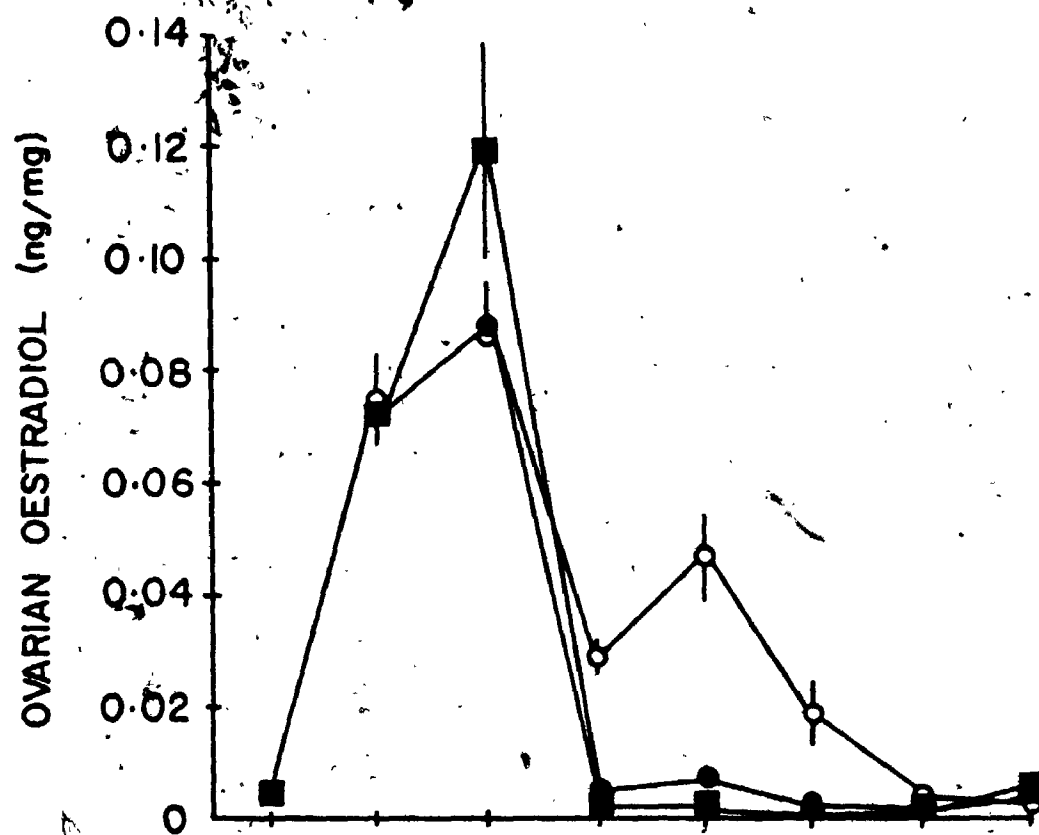
Serum progesterone and oestradiol concentrations in control, SOV and SOV a/s treated rats. PMSG was injected on day -2 and a/s on day 0. Results are mean  $\pm$  S.E.M. for 10 rats per treatment per day. Standard errors not shown fall within the points as drawn.





# FIGURE 9

Ovarian oestradiol content and concentrations in control, SOV and SOV a/s treated rats. PMSG was injected on day -2 and a/s. on day 0. Results are mean  $\pm$  S.E.M. for 10 rats per treatment per day. Standard errors not shown fall within the points as drawn.



difference between SOV untreated and SOV a/s-treated rats was significant ( $P = 0.025$ ). Serum oestradiol concentrations showed a pattern similar to ovarian content, there being an increase from 30 pg/ml on day -2 to 90 pg/ml in control and 170 pg/ml in SOV on day 0 (Figure 8). After ovulation values in control rats returned to those seen at the start of the experiment whereas in SOV a/s rats the concentration also dropped but was significantly higher ( $P = 0.025$ ) until day 5. SOV rats showed a small drop of serum oestradiol concentrations to 100 pg/ml on day 1 followed by a second peak of 160 pg/ml on day 2, after which values declined to close to those of control rats by day 5. The difference between SOV and SOV a/s rats was significant ( $P = 0.025$ ).

Concentrations of steroids in serum and ovaries on day 8 are shown in Table 8. Comparing days 5 and 8 in all three superovulatory treatments there were no significant differences except in total ovarian oestrogen content which declined significantly ( $P < 0.001$ ) in SOV rats. In control animals serum oestradiol concentrations declined significantly ( $P < 0.01$ ) whereas total ovarian progesterone content and ovarian progesterone/mg wet tissue increased significantly ( $P < 0.01$ ).

On day 8 both oestradiol and progesterone concentrations were significantly lower ( $P < 0.01$ ) in the serum of control rats than in the serum of all three superovulatory treatments (Table 8). Total ovarian oestradiol content was significantly higher in SOV a/s rats than in control or SOV rats ( $P < 0.05$ ). When expressed as pg/mg, concentrations in SOV rats were significantly lower than in SOV a/s animals ( $P < 0.05$ ), whereas the concentration in control rats was significantly higher ( $P < 0.01$ ) than in the three superovulatory treatments. Total ovarian progesterone content was significantly higher ( $P < 0.01$ ) in SOV rats than

TABLE 8

Steroid Concentrations and Content on Day 8 after Ovulation (mean  $\pm$  S.E.M.;  
for details of treatments see text; n = 20 for each treatment)

	Treatment		
	Control	SOV	SOV a/s E <sub>2</sub>
Total ovarian oestradiol (pg)	205 $\pm$ 13 <sup>a</sup>	212 $\pm$ 10 <sup>a</sup>	285 $\pm$ 26 <sup>b</sup>
Ovarian oestradiol (pg/mg)	5.2 $\pm$ 0.3 <sup>a</sup>	1.5 $\pm$ 0.1 <sup>b</sup>	2.3 $\pm$ 0.3 <sup>c</sup>
Serum oestradiol (pg/ml)	20 $\pm$ 2 <sup>a</sup>	36 $\pm$ 3 <sup>b</sup>	53 $\pm$ 5 <sup>b</sup>
Total ovarian progesterone (ng)	1220 $\pm$ 80 <sup>a</sup>	3120 $\pm$ 370 <sup>b</sup>	2220 $\pm$ 190 <sup>b,c</sup>
Ovarian progesterone (ng/mg)	30.6 $\pm$ 1.9 <sup>a</sup>	22.1 $\pm$ 2.7 <sup>b</sup>	20.5 $\pm$ 1.4 <sup>b</sup>
Serum progesterone (ng/ml)	55 $\pm$ 4 <sup>a</sup>	254 $\pm$ 25 <sup>b</sup>	217 $\pm$ 21 <sup>b</sup>
			40 $\pm$ 3 <sup>b</sup>
			1510 $\pm$ 140 <sup>a,c</sup>
			12.6 $\pm$ 1.1 <sup>c</sup>
			232 $\pm$ 20 <sup>b</sup>

a, b, c within each variable, means with the same superscript are not significantly different

in control and SOV a/s  $E_2$  animals, and levels in SOV a/s rats were also significantly higher ( $P < 0.01$ ) than control values. When expressed as ng/mg concentrations in control rats were significantly higher ( $P < 0.05$ ) than in the three superovulatory treatments. Concentrations in SOV a/s  $E_2$  rats were significantly lower ( $P < 0.05$ ) than in SOV and SOV a/s rats.

Table 9 shows the serum and ovarian concentrations of oestradiol and progesterone on day 20 for pregnant rats only. There was no significant difference in serum oestradiol concentrations between treatments, whereas total ovarian oestradiol content and oestradiol/mg wet tissue in control rats were significantly lower ( $P < 0.002$ ) than in both the superovulatory treatments. Serum progesterone concentrations were significantly lower ( $P < 0.01$ ) in control animals than in SOV a/s or SOV a/s  $E_2$  rats and levels in SOV a/s rats were also significantly higher than those in SOV a/s  $E_2$  rats ( $P < 0.02$ ). Total ovarian progesterone content was significantly lower in control animals ( $P < 0.001$ ) than in either of the other two groups, whereas there was no significant difference between the three groups when the concentrations were expressed per mg wet tissue.

Between days 8 and 20 concentrations of oestradiol in both serum and ovaries rose significantly in all groups ( $P < 0.01$ ). Serum progesterone levels were unchanged in all groups over this period, whereas total ovarian progesterone was significantly increased in both SOV a/s and SOV a/s  $E_2$  groups ( $P < 0.001$ ). When expressed as ng/mg wet tissue, only control and SOV a/s  $E_2$  rats had significantly higher ovarian progesterone concentrations on day 20 compared to day 8 ( $P < 0.001$  and  $P < 0.05$ , respectively).

TABLE 9

Steroid Concentrations and Content on Day 20 of Pregnancy (mean  $\pm$  S.E.M.;  
for details of treatments see text;  
 $n = 24, 10$  and  $7$  for control, SOV a/s and SOV a/s  $E_2$  rats respectively)

	Treatment		
	Control	SOV a/s	SOV a/s $E_2$
Total ovarian oestradiol (pg)	580 $\pm$ 37 <sup>a</sup>	1350 $\pm$ 770 <sup>b</sup>	1270 $\pm$ 160 <sup>b</sup>
Ovarian oestradiol (pg/mg)	8.2 $\pm$ 0.6 <sup>a</sup>	4.7 $\pm$ 0.3 <sup>b</sup>	4.6 $\pm$ 0.4 <sup>b</sup>
Serum oestradiol (pg/ml)	74 $\pm$ 5 <sup>a</sup>	91 $\pm$ 6 <sup>a</sup>	83 $\pm$ 9 <sup>a</sup>
Total ovarian progesterone (ng)	1300 $\pm$ 92 <sup>a</sup>	4640 $\pm$ 420 <sup>b</sup>	4850 $\pm$ 870 <sup>b</sup>
Ovarian progesterone (ng/mg)	18.4 $\pm$ 1.3 <sup>a</sup>	16.0 $\pm$ 1.4 <sup>a</sup>	18.5 $\pm$ 3.3 <sup>a</sup>
Serum progesterone (ng/ml)	72 $\pm$ 6 <sup>a</sup>	262 $\pm$ 26 <sup>b</sup>	164 $\pm$ 28 <sup>c</sup>

<sup>a, b, c</sup> within each variable, means with the same superscript are not significantly different.

#### 6.2.2.4 Cross Reactivity of PMSG: PMSG cross reacts

with the LH antibody used in rat LH assays in this laboratory (Figure 10); 0.076 i.u. PMSG displaced 50% of iodinated LH from the antibody; this displacement being equivalent to 90 ng LH (NIH-LH-S19). As a result of this cross reactivity no LH assays were performed.

6.2.2.5 Histology: Plates 1 and 2 each show an example of a central cross-section of an ovary from a SOV (Plate 1) and a SOV a/s rat (Plate 2) on day 3 of pregnancy. The distribution of follicles by size on days 2 and 3 is shown in Figures 11 and 12. Each bar represents the mean ( $\pm$  S.E.M.) of the total number of follicles in each size class in the central 10 sections of 5 ovaries. Following injection of a/s on day 0 there was no significant difference in the number of follicles in each size class on day 2, whereas on day 3 the number of small ( $< 100 \mu\text{m}$ ) and medium ( $100-500 \mu\text{m}$ ) follicles was not significantly different, but the number of large follicles ( $> 500 \mu\text{m}$ ) was significantly reduced ( $P < 0.001$ ).

### 6.3 Superovulation with FSH Infusion

#### 6.3.1 Methods

Alzet mini-osmotic pumps model 2001 (Alza Corp., Palo Alto, CA) were filled with a crude FSH preparation, extracted from porcine pituitaries by the method of Steelman et al. (1953). The concentration was calculated to infuse  $250 \mu\text{g/day}$ . As measured by radioreceptor

FIGURE 10

Cross reactivity of PMSG with LH antiserum.

(B/T = Bound counts per minute/Total counts per minute).



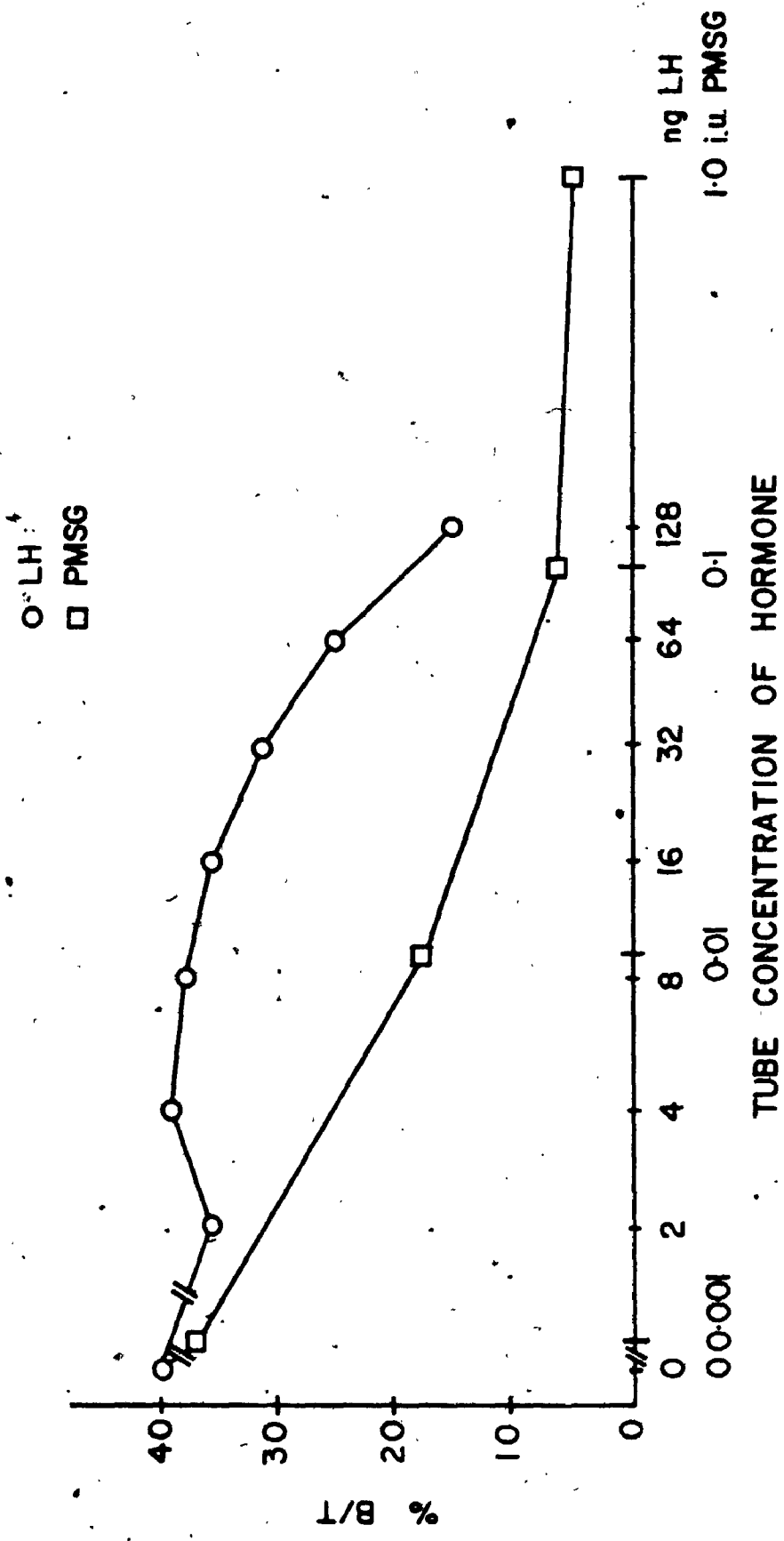


PLATE 1

Central cross section of an ovary on day 3 of pregnancy  
following superovulation with 40 i.u. PMSG (X20).

PLATE 2

Central cross section of an ovary on day 3 of pregnancy  
following superovulation with 40 i.u. PMSG + a/s (X20).

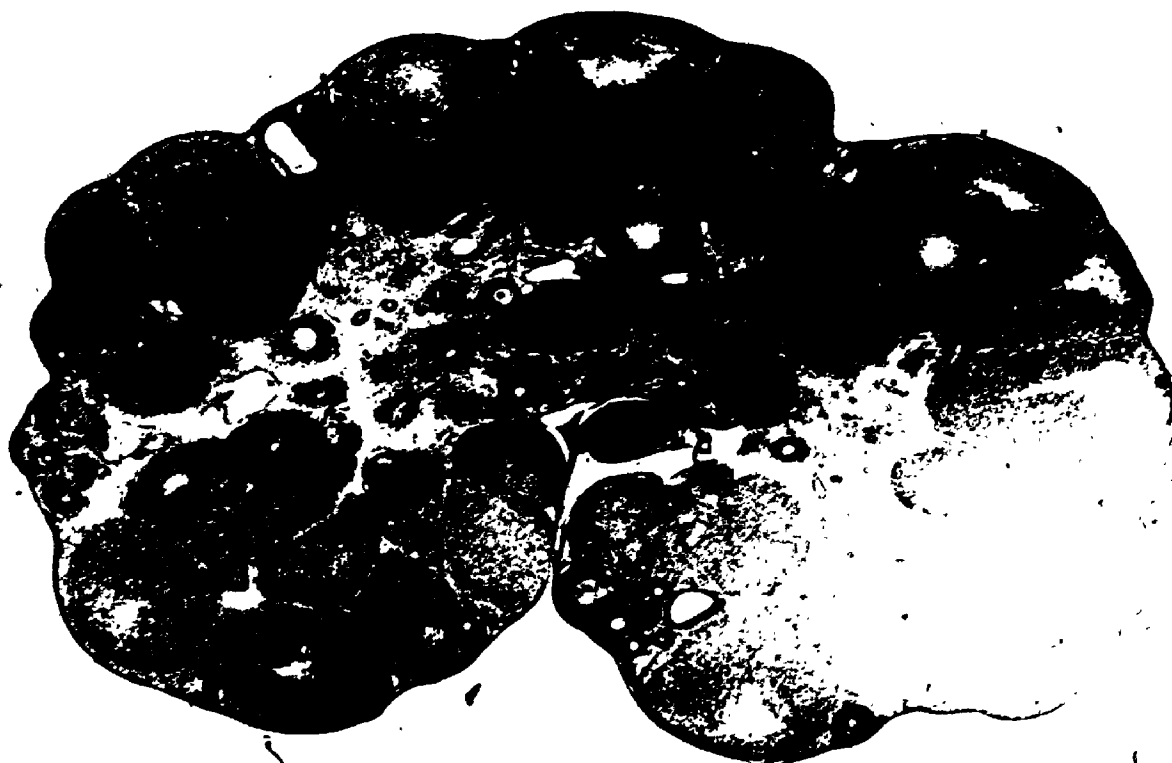


FIGURE 11

Size of follicles in central 10 ovarian sections on day 2 of pregnancy after superovulation with 40 i.u. PMSG on day -2  $\pm$  a/s on day 0.

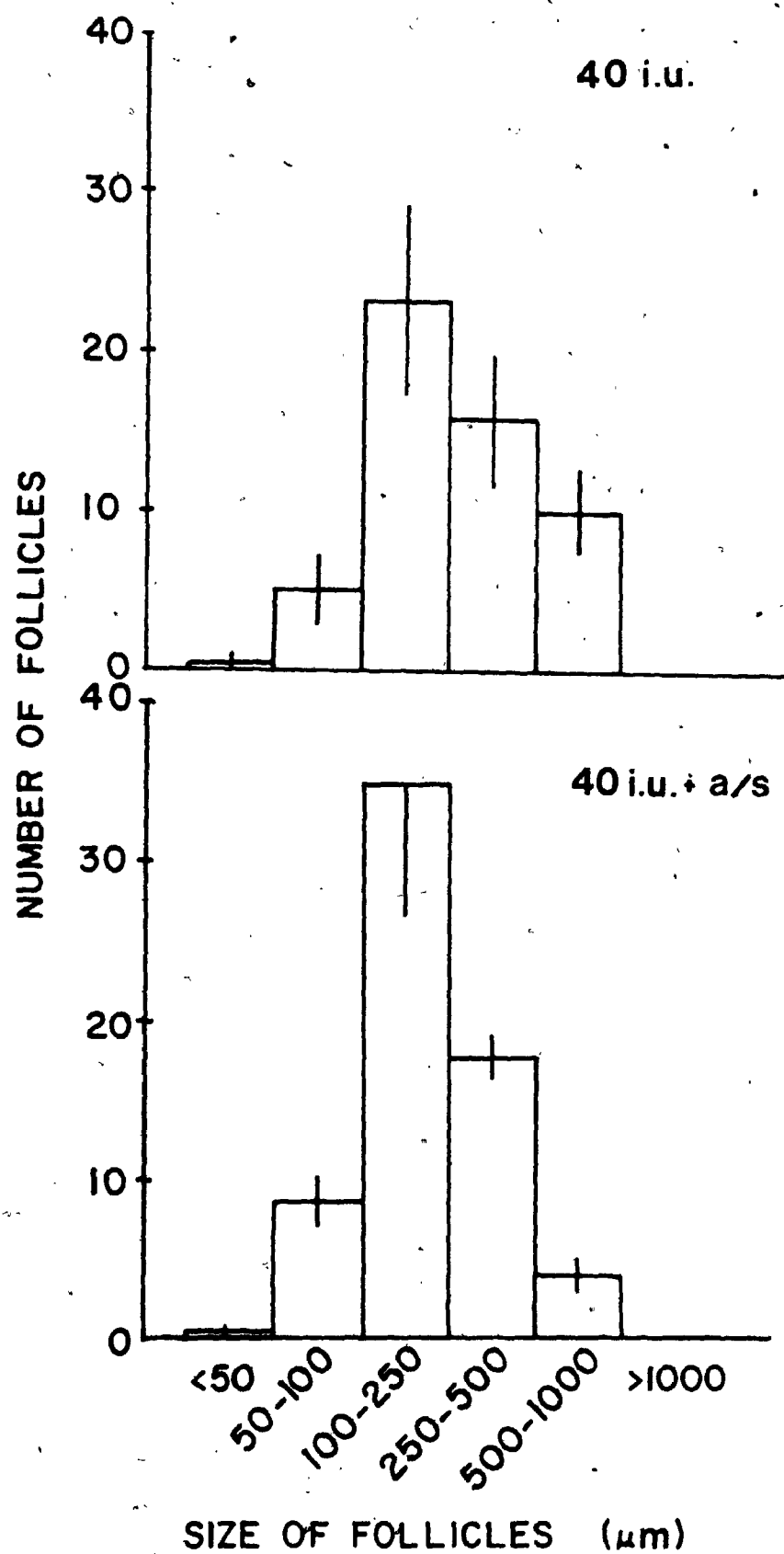
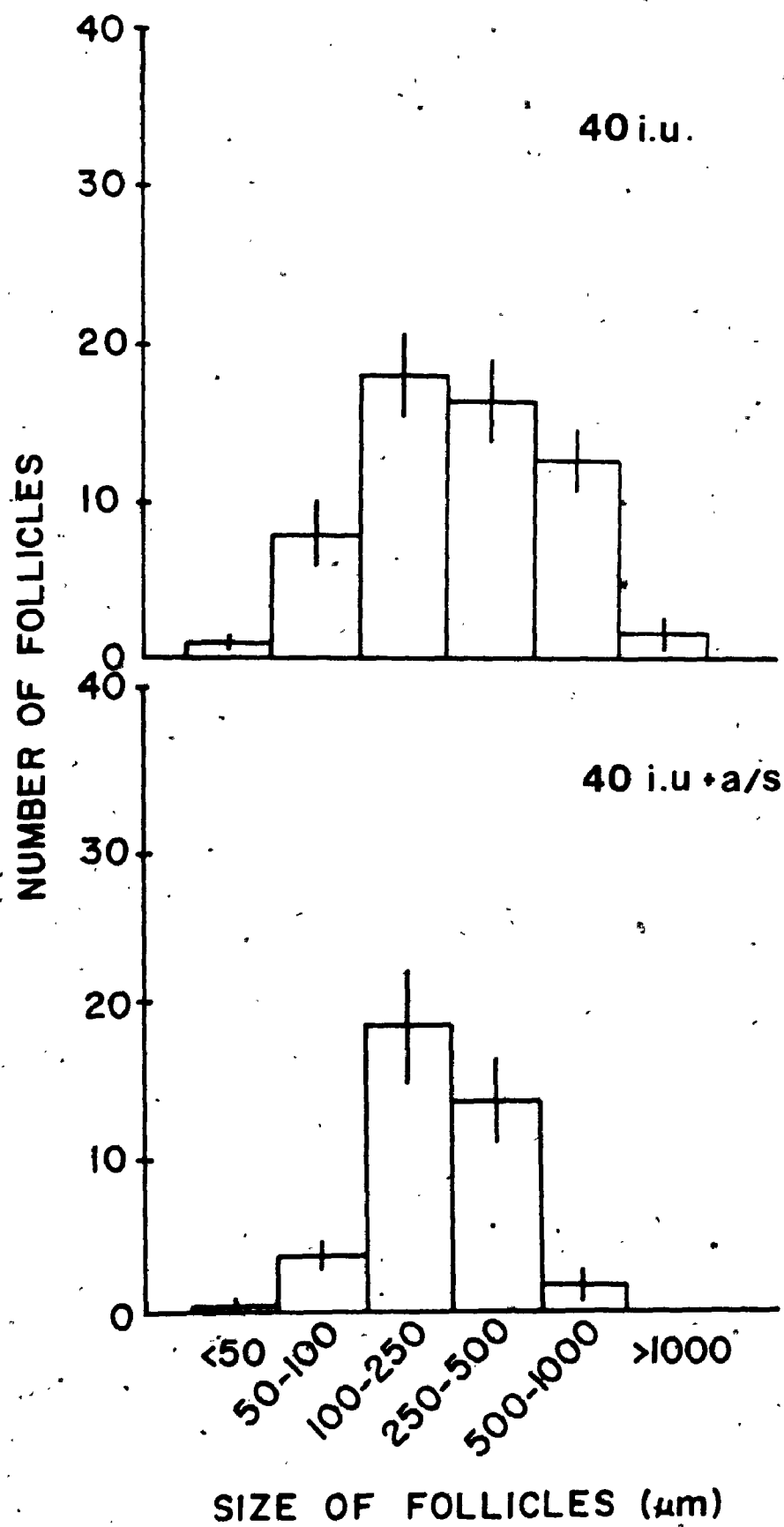


FIGURE 12

Size of follicles in central 10 ovarian sections on day 3 of pregnancy after superovulation with 40 i.u. PMSG on day -2  $\pm$  a/s on day 0.



assays the potency of FSH was 36% that of FSH-P (Burns Biotech, Oakland, CA; Lot No. 519E78) and LH contamination was  $\sim 4 \mu\text{g}$  LH (NIH-LH-B4)/100  $\mu\text{g}$  FSH (D.T. Armstrong, personal communications). Various procedures (Figure 13) were tested to determine the optimum method for producing superovulation. Pumps were either filled and immediately implanted (Groups B, C and D), or were filled the previous evening and primed overnight in 0.9% saline at room temperature (Group A). On day -2, at the times shown in Figure 13 (0800 h, Groups A and B; 0500 - 0600 h, Groups C and D), the pumps were implanted s.c. in immature rats using light ether anaesthesia. The pumps were removed at the times shown on day 0 (1700 h, Groups A, B and C; 2200 h, Group D) and LH (NIH-LH-B8; 10  $\mu\text{g}$  in 0.2 ml 0.9% NaCl) was injected i.p. in some rats at 1700 h. Animals were sacrificed by cervical dislocation on the morning of day 1, ovarian and uterine weights were recorded and the oviducts were flushed to recover oocytes. Any oocytes not surrounded by cumulus cells were considered to be greater than 20 h post ovulation (McCormack and Bennin, 1970).

Rats in Group D and some rats in Group A (-LH) were mated overnight of day 0 and were sacrificed on days 1, 2, 3, 5, 8 and 20 of pregnancy. The ovaries were weighed on each day, immediately transferred to chilled ethanol and ovarian steroid levels were later assayed, as described previously in this chapter. On days 1-5 uterine weights were also recorded and the oviducts and uteri were flushed to recover embryos. On day 8 implantation sites were counted and the total implantation site weight was recorded. On day 20 the number of foetuses and placentae were counted, together with the number of live foetuses. Total live foetal and placental weights were recorded.

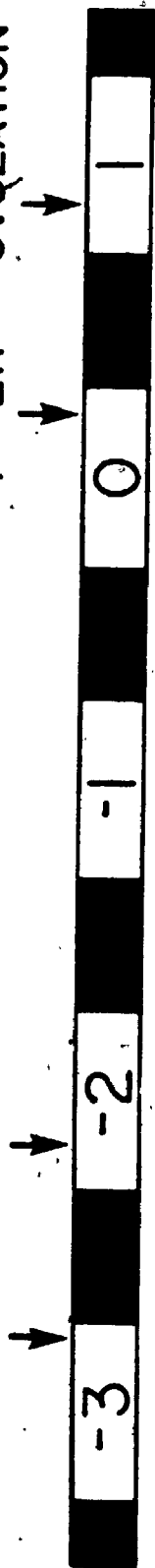


FIGURE 13

Schematic representation of the procedures used to determine the optimum conditions for the induction of superovulation by infusion of FSH. (Numbers represent the day of the experiment, where day 1 = first day of pregnancy; dark areas are dark periods, 1900 - 0500 h).

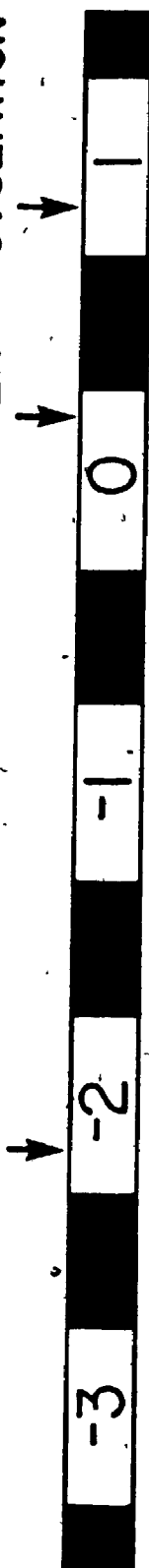
PUMPS PUMPS  
FILLED INSERTED

PUMPS OUT CHECK FOR  
+/- LH OVULATION



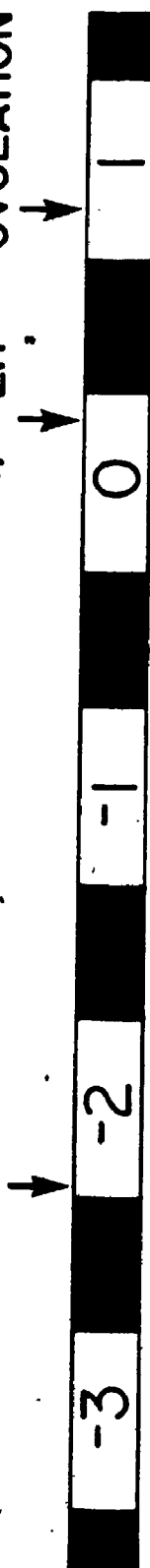
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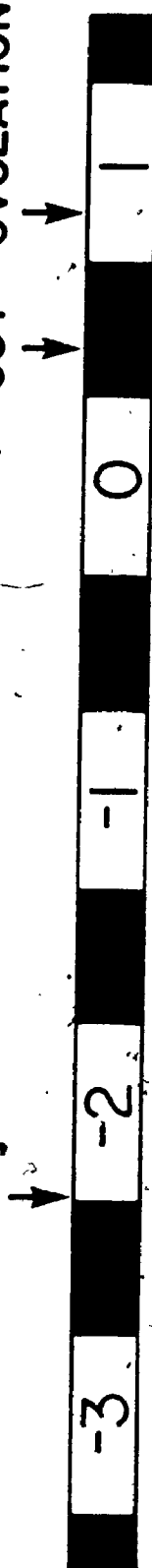
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The proportion of rats pregnant on each day was compared using a  $\chi^2$  test. The mean recovery rates of oocytes, embryos, implantation sites and foetuses against time were analyzed by linear regression; ovarian and uterine weights and ovarian steroid concentrations and content were compared using one-way analysis of variance and Duncan's New Multiple Range Test. When necessary to remove heterogeneity of variance, data were logarithmically transformed.

### 6.3.2 Results

The results of the different procedures used to induce superovulation with FSH infusion are given in Table 10. With the exception of Group C + LH (n = 5) it was not possible to produce ovulation in 100% of rats. In some animals premature ovulation occurred and degenerate oocytes and oocytes without attached cumulus cells were recovered.

Following mating the percentage of rats pregnant on days 1, 2, 3, 5, 8 and 20 is shown in Figure 14. There was no significant difference in the percentage pregnant between days. Figure 15 shows the mean embryo recovery rates on days 1, 2, 3 and 5 with values ranging from  $20.8 \pm 4.0$  on day 1 to  $13.8 \pm 2.8$  on day 5. On day 8 a mean of  $25.3 \pm 4.7$  implantation sites was observed and on day 20  $15.0 \pm 5.4$  live foetuses were recovered from those rats which were pregnant. There was no significant difference between these days in the mean number of embryos, implantation sites or foetuses.

Figure 16 shows the changes in ovarian and uterine wet weights over the period day -2 to day 5. There was no significant

TABLE 10

Ovulation Rates after Superovulation by Continuous Infusion of FSH  
(for details of procedures see Figure 12)

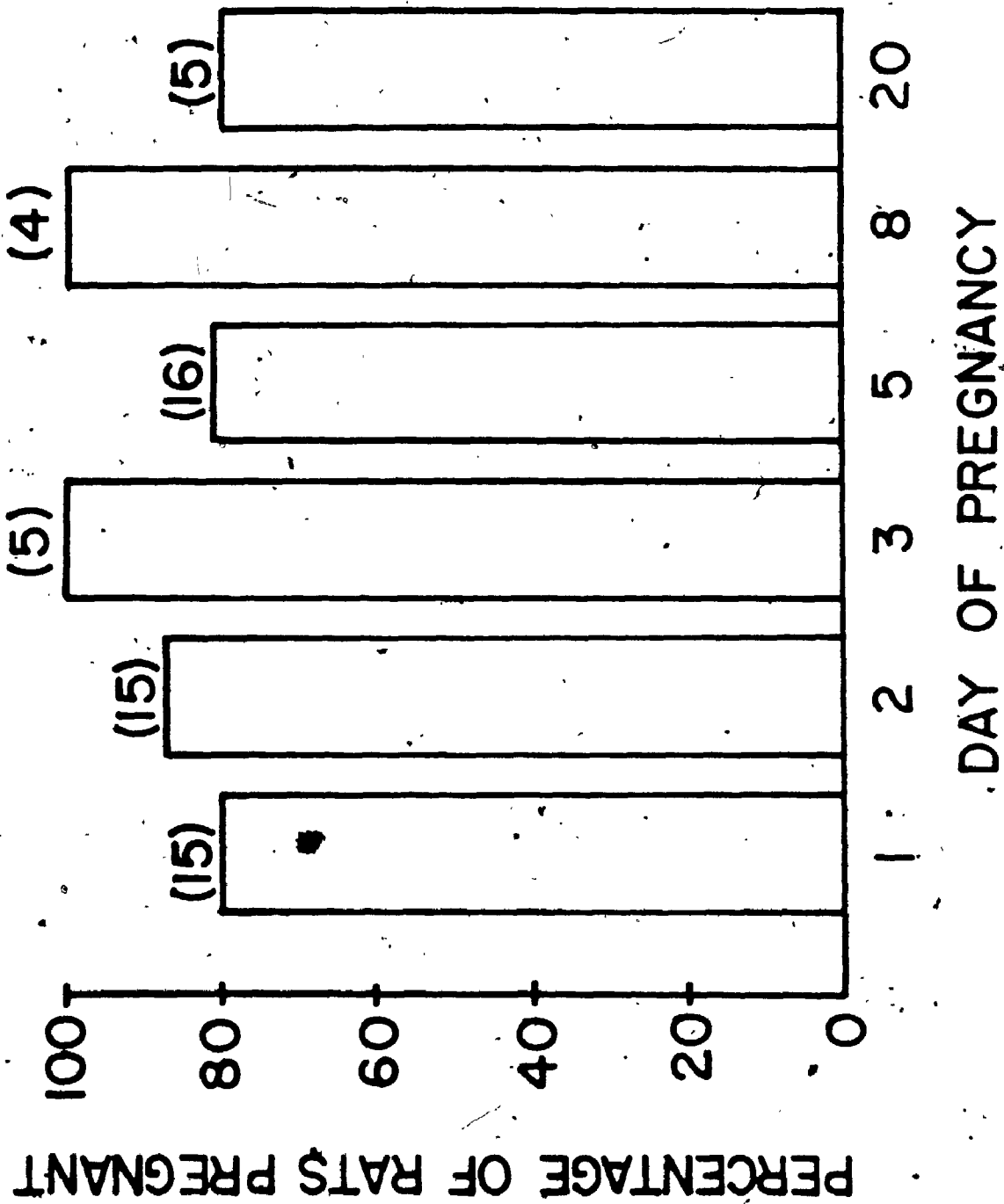
<u>Procedure</u>	<u>LH</u>	<u>Proportion of Rats Ovulating</u>	<u>Proportion of Rats with Fresh Ovulations</u>	<u>Number of Ovulations (mean <math>\pm</math> S.E.M.*)</u>
A	+	4/5	4/5	34.0 $\pm$ 3.5
	-	9/11	6/11	33.7 $\pm$ 4.9
B	+	4/5	2/5	25.5 $\pm$ 5.5
	-	1/5	1/5	17.0
C	+	5/5	5/5	23.8 $\pm$ 1.7
	-	3/5	3/5	28.7 $\pm$ 7.1
D	-	14/16	9/16	25.3 $\pm$ 3.1

\*only rats with fresh ovulations included.

FIGURE 14

Percentage of rats pregnant following superovulation induced by infusion of FSH. (Numbers in brackets are the number of rats sacrificed on each day). Days 1, 2 and 5 include rats from groups A and D; days 8 and 20 are rats from group D only.

14



# FIGURE 15

Embryo recovery rates (mean  $\pm$  S.E.M.) in rats superovulated by infusion of FSH. Further details of the infusion are given in the text. (For comparison data from the previous experiments with PMSG are included).

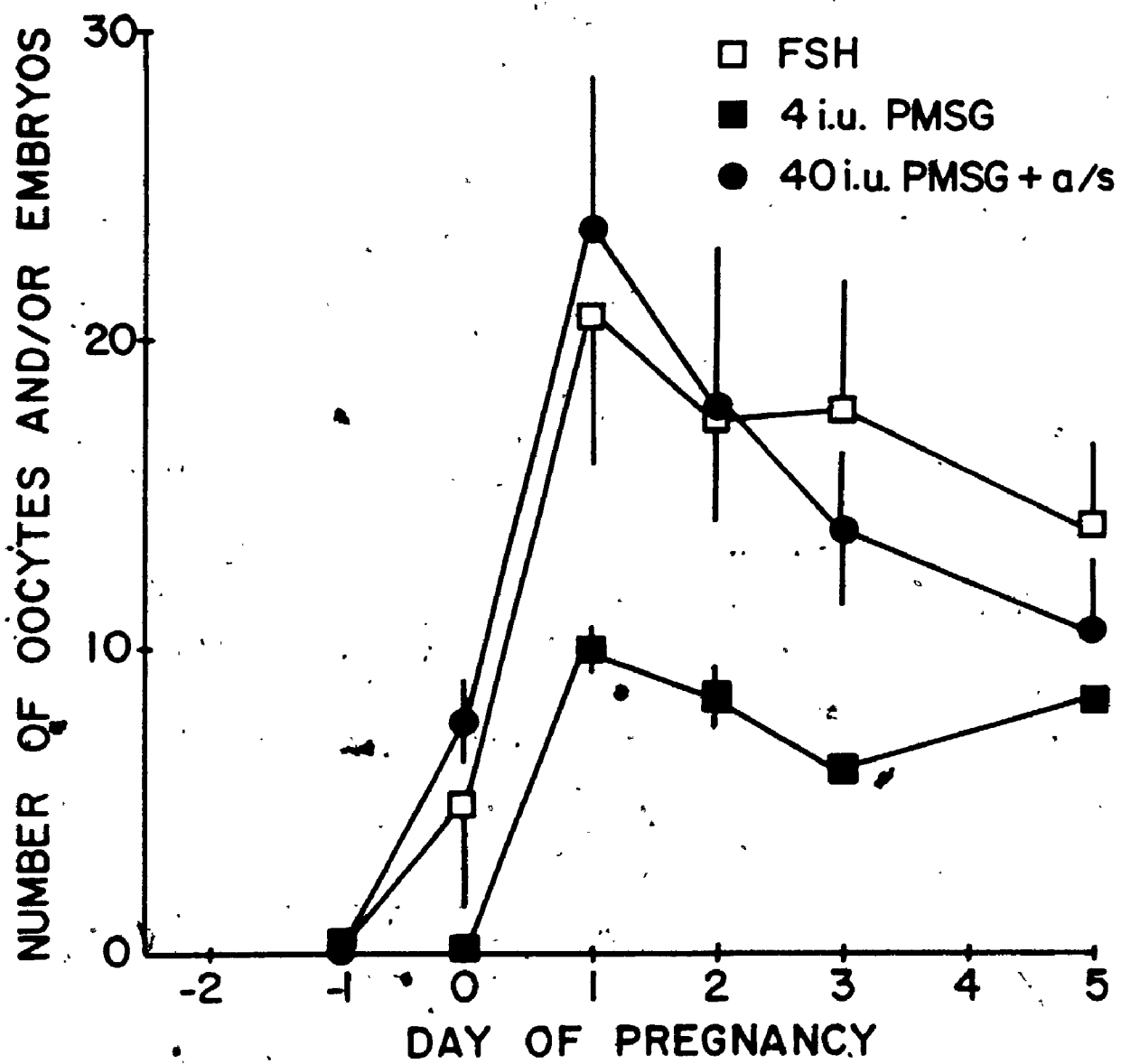
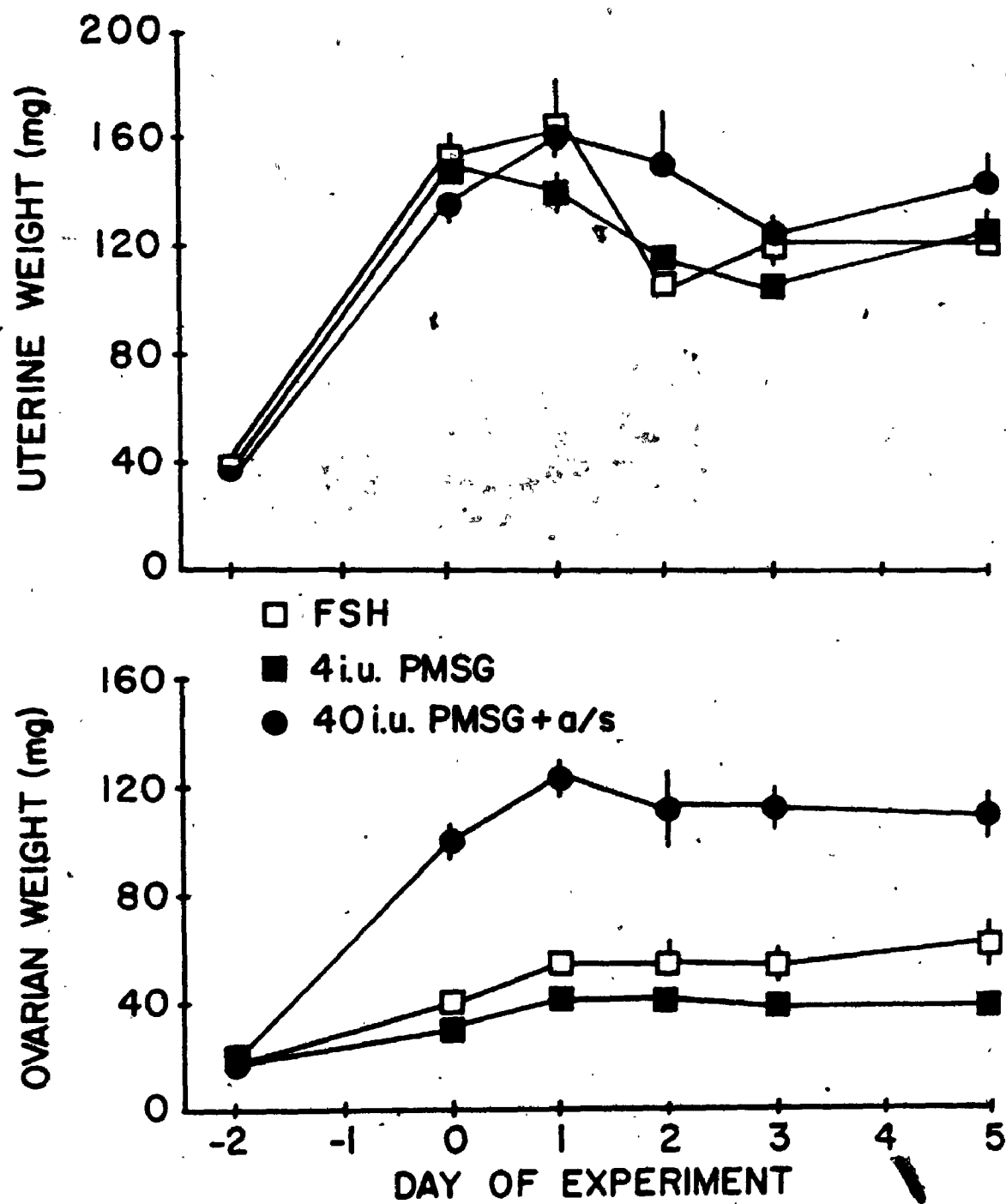




FIGURE 16

Ovarian and uterine weights in rats superovulated by infusion of FSH. Results are mean  $\pm$  S.E.M. Standard errors not shown fall within the points as drawn. (For comparison data from previous experiments with PMSG are included).



1  
difference in ovarian weight over the period day 1 to day 5, whereas values rose significantly from 20 mg on day -2 to 54 mg on day 1. Uterine weight on day 1 (163 mg) was significantly higher ( $P < 0.01$ ) than on any other day. On day 8 the ovarian weight of  $61.9 \pm 7.9$  mg was not significantly different from that observed on day 5, whereas on day 20 the ovarian weight of  $116.0 \pm 25.3$  mg was significantly higher than on any other day of pregnancy ( $P < 0.01$ ).

The mean weight of implantation sites on day 8 was  $14.5 \pm 1.5$  mg (compared to 4 i.u. 17.6 mg and 40 i.u. + a/s 13.6 mg in the previous experiments) and foetal and placental weights on day 20 were  $1.8 \pm 0.2$  g and  $385 \pm 44$  mg respectively (compared to 4 i.u. 1.8 g and 473 mg and 40 i.u. + a/s 1.1 g and 307 mg respectively).

Ovarian levels of oestradiol and progesterone, expressed both as total ovarian concentration and per mg ovarian tissue, are shown in Figures 17 and 18 (days 1-5) and Table 11 (days 8 and 20). Total ovarian oestradiol content on day 0 was 2.6 ng which was significantly higher ( $P < 0.01$ ) than the content on subsequent days of pregnancy. When expressed per mg ovarian tissue it was also significantly higher ( $P < 0.01$ ) on day 0. Total ovarian oestradiol content and ovarian oestradiol per mg tissue were also higher on day 20 than on any other day of pregnancy from day 2 onwards ( $P < 0.01$ ). Total ovarian progesterone content rose from 80 ng on day 0 to 1,500 ng by day 8, although the content was not significantly different between days from day 3 onwards. A similar pattern was seen when results were expressed per mg ovarian tissue.

Following superovulation induced by infusion of FSH, the

# FIGURE 17

Ovarian oestradiol content and concentrations in rats superovulated by infusion of FSH. Results are mean  $\pm$  S.E.M. Standard errors not shown fall within the points as drawn. (For comparison data from previous experiments with PMSG are included).

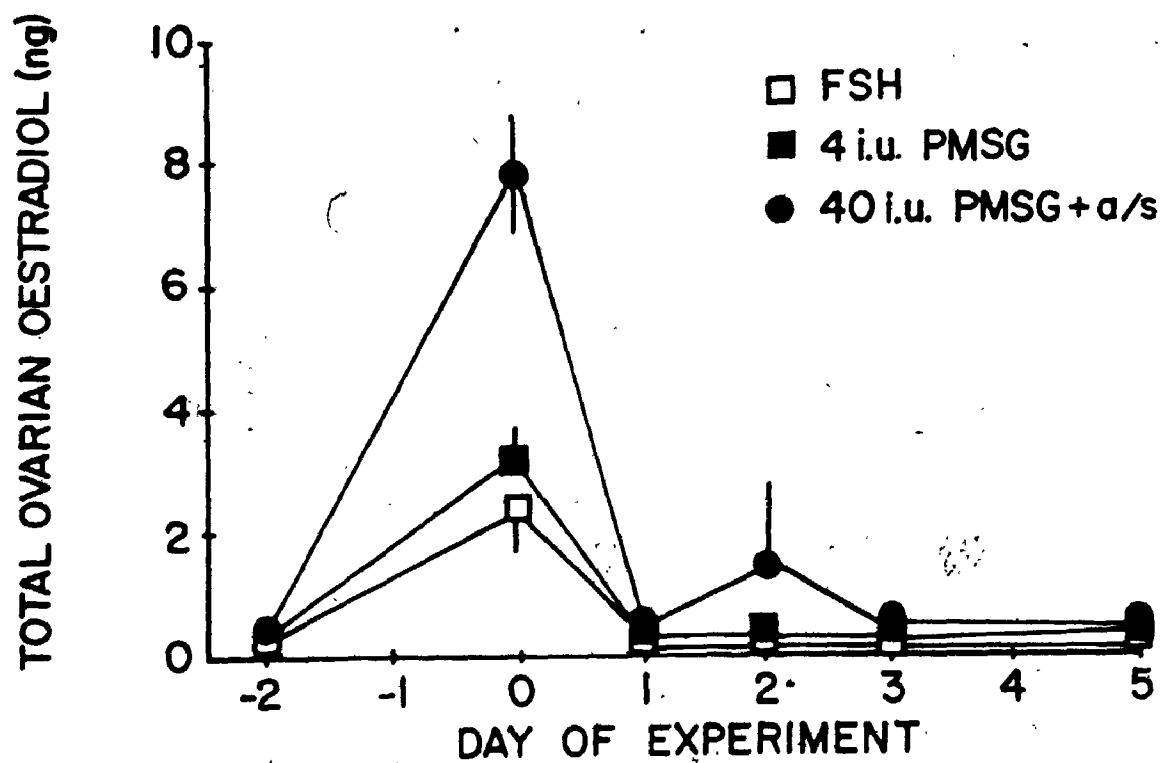
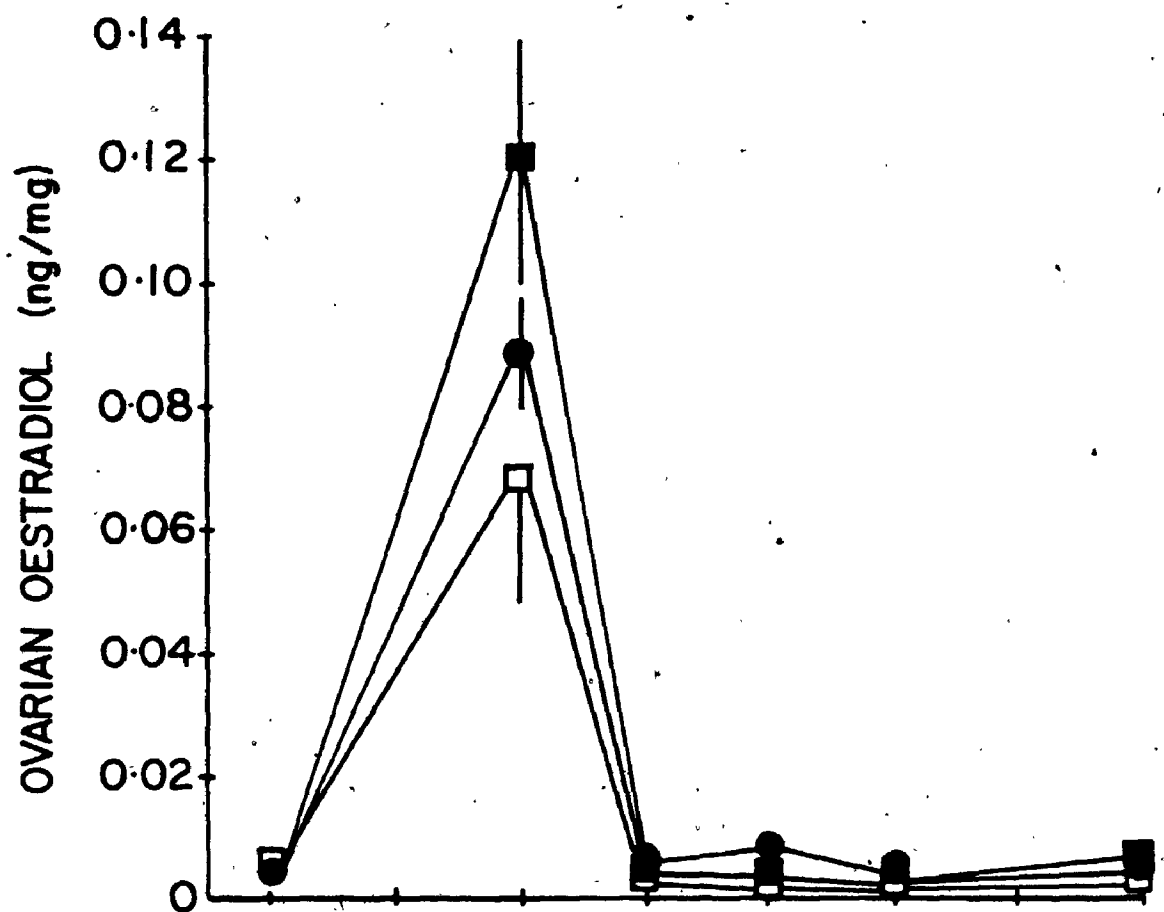


FIGURE 18

Ovarian progesterone content and concentrations in rats superovulated by infusion of FSH. Results are mean  $\pm$  S.E.M. Standard errors not shown fall within the points as drawn. (For comparison data from previous experiments with PMSG are included).

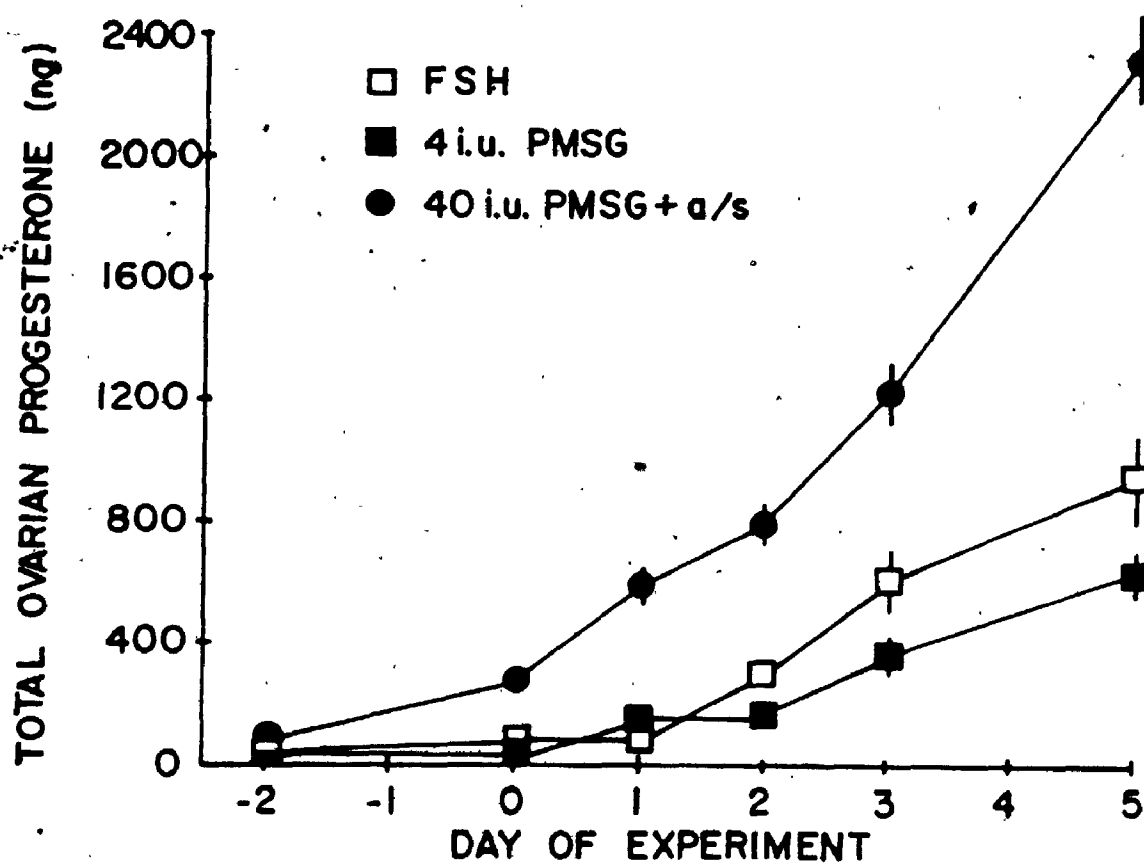
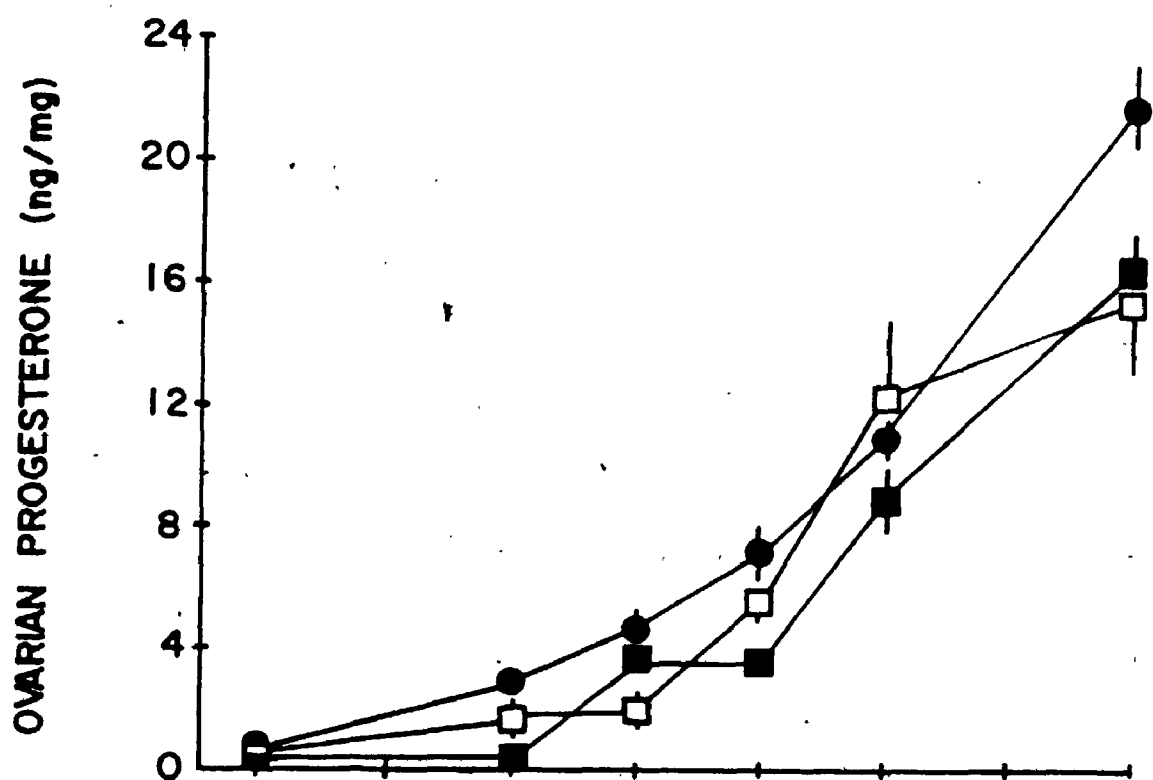


TABLE 11

Ovarian Oestradiol and Progesterone Content and Concentrations  
on Days 8 and 20 of Pregnancy after Superovulation  
by Continuous Infusion of FSH (mean  $\pm$  S.E.M.)

	<u>Day 8</u>	<u>Day 20</u>
Total ovarian oestradiol (pg)	122 $\pm$ 27	943 $\pm$ 291
Ovarian oestradiol (pg/mg)	1.9 $\pm$ 0.4	6.9 $\pm$ 0.6
Total ovarian progesterone (ng)	1460 $\pm$ 360	1260 $\pm$ 320
Ovarian progesterone (ng/mg)	22.8 $\pm$ 3.8	9.9 $\pm$ 1.3



weights of ovaries and uteri and ovarian concentrations and content of oestradiol and progesterone were closer to those following induction of ovulation with 4 i.u. PMSG than to values following superovulation with 40 i.u. + a/s (Figures 16 - 18). Embryo recovery rates were higher than either PMSG treatment from day 5 onwards (Figure 19). The percentage of rats pregnant was equal to control 4 i.u. rats and greater than 40 i.u. + a/s rats from day 8 onwards (Figure 20).

#### 6.4. Discussion

Several authors have shown changes in ovarian weight in immature rats following injection of PMSG at both low (Sawamoto and Sasamoto, 1973; Parker et al., 1976; Koštyk et al., 1978; Miller and Armstrong, 1981b) and superovulatory doses (Bell and Lunn, 1966; Lunn and Bell, 1968; Zarrow and Brown-Grant, 1964; Wilson et al., 1974; Miller and Armstrong, 1981b); absolute values may vary between studies, but the results are generally similar to those observed in the present study. Rats injected with the control dose of PMSG (4 i.u.) show similar increases to those observed during the first spontaneous oestrus (Osman, 1975; Andrews et al., 1980), whereas superovulation with PMSG resulted in a much greater increase. Ovulation of a similar number of oocytes occurred following superovulation with either 40 i.u. PMSG or FSH infusion, but the increase in ovarian weight was much lower following FSH infusion (Figure 16). This suggests that PMSG is either stimulating the development of a considerable number of follicles which do not ovulate, or increased



FIGURE 19

Recovery of oocytes/blastocysts/implantation sites/foetuses (mean number recovered  $\pm$  S.E.M.) on days 1, 5, 8 and 20 of pregnancy, following induction of ovulation with 4 i.u. or 40 i.u. PMSG + a/s or by infusion of FSH. The data are compiled from different experiments; for numbers of rats in each treatment see Tables 3, 4 and 5 and Figure 14.

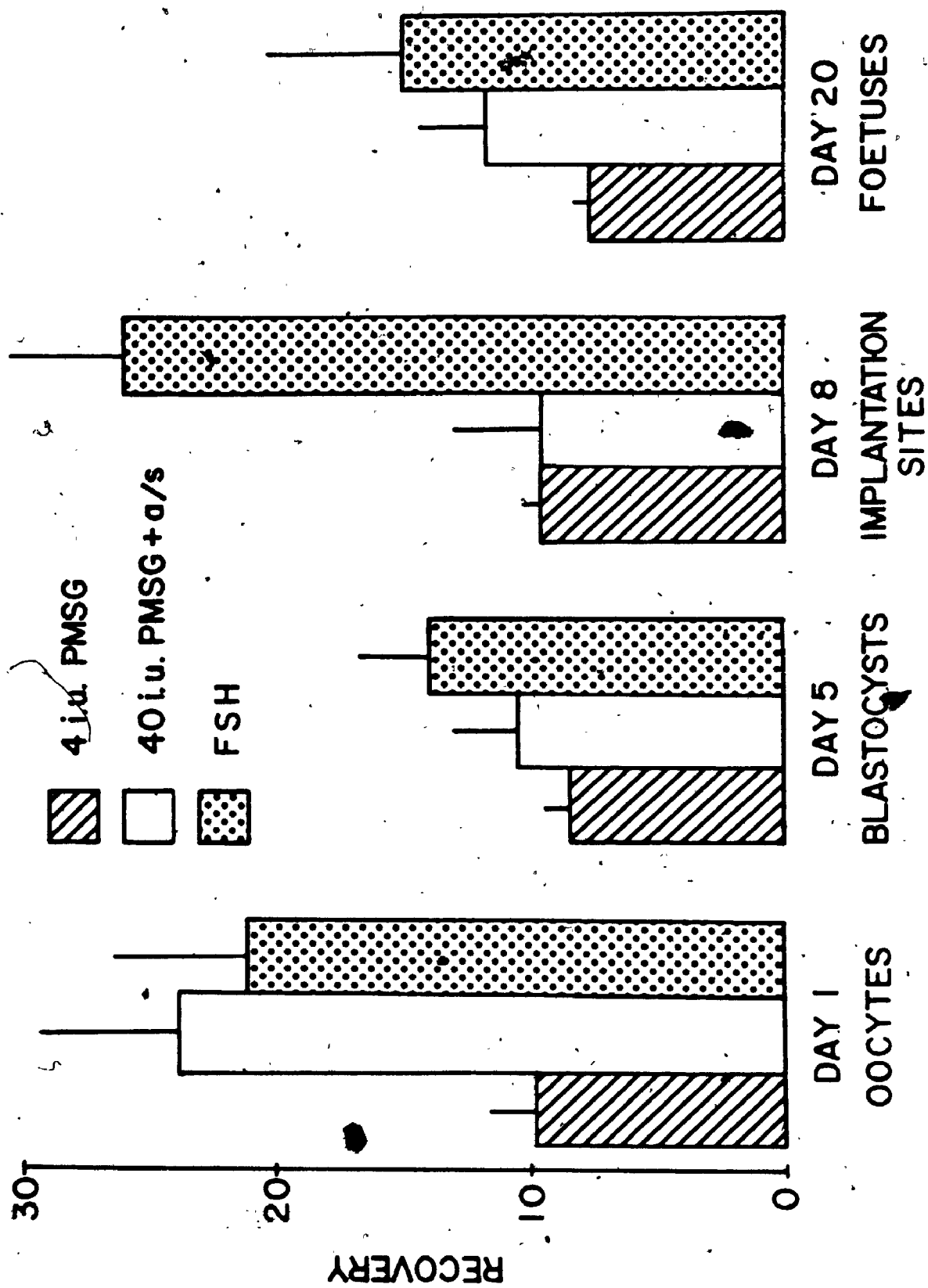
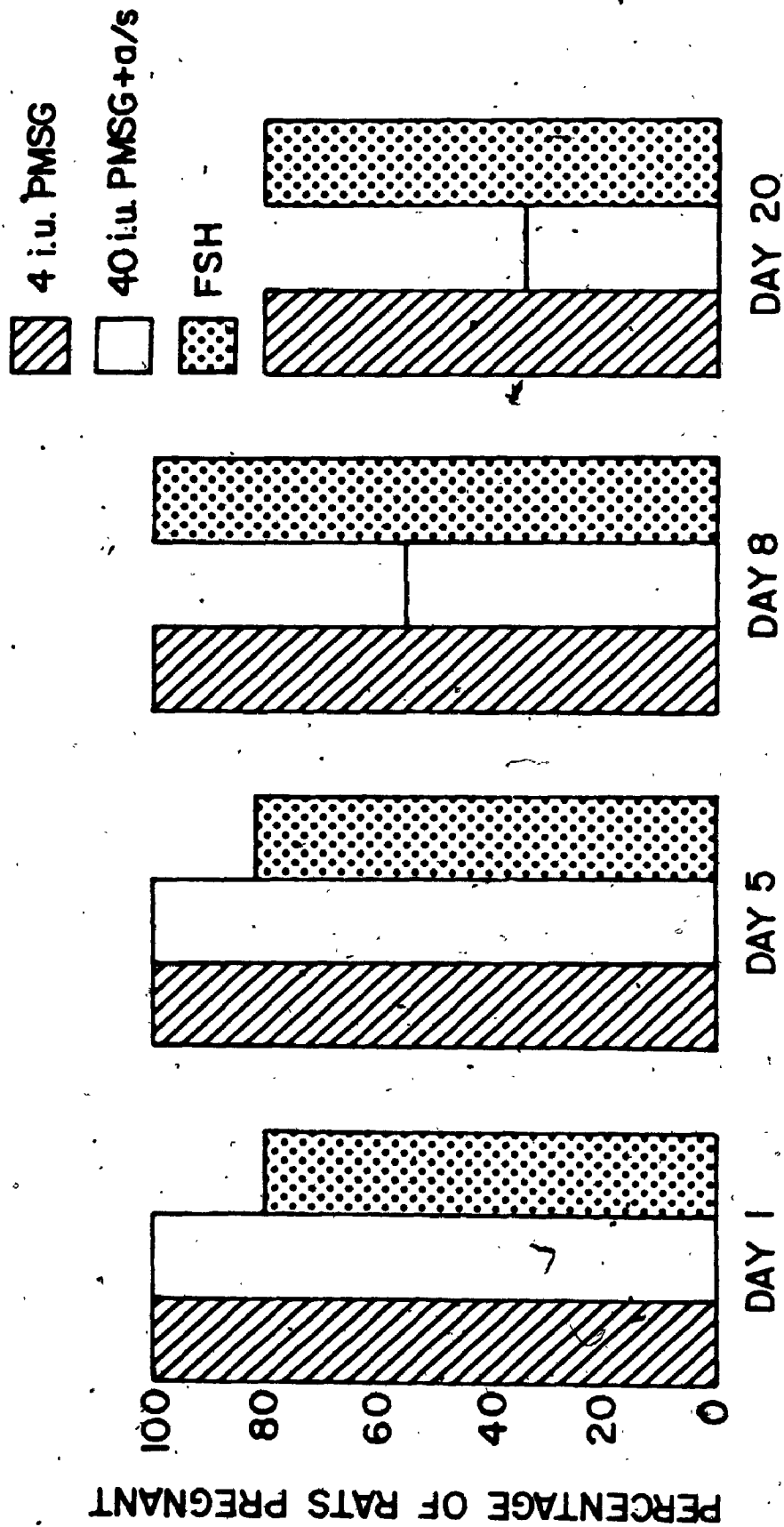


FIGURE 20

Percentage of rats pregnant on days 1,5,8 and 20 of pregnancy following induction of ovulation with 4 i.u. or 40 i.u. PMSG + a/s or by infusion of FSH.



development of extrafollicular ovarian tissue; alternatively, the same number of follicles might be stimulated to a greater degree.

Since uterine growth in both control and SOV rats is similar between days -2 and 0 (Figure 6) it has been suggested that serum oestradiol levels in all animals were sufficient to elicit a maximum growth response (Miller and Armstrong, 1981b). Alternatively, since the present experiments show that serum oestradiol concentrations are higher in SOV rats, compared to controls, only between days -1 and 0 (Figure 8), this may be insufficient time to elicit greater growth in the superovulated rats. The continued growth seen in the SOV group after day 1 may reflect the persistently high serum oestradiol concentrations in this group (Figure 8).

The steroidogenic properties of PMSG in the immature rat have been well documented (Zarrow et al., 1971; Endersby et al., 1973; Wilson et al., 1974; Parker et al., 1976; Sashida and Johnson, 1976) and PMSG has uniformly been found to cause a large increase in serum concentrations of both oestradiol and progesterone, similar to those observed in the present study. As a result of the modification of ovarian enzyme activity, which results in a shift to oestradiol production (Suzuki et al., 1978), oestradiol concentrations peak ~ 48 h after PMSG (Figures 8 and 9). Progesterone levels rise dramatically in the early evening prior to the LH surge (Zarrow et al., 1971) and ovulation (Wilson et al., 1974; Parker et al., 1976), following an increase in testosterone production (Bauminger et al., 1977). In the present studies this peak of progesterone was not recorded due to 24 h sampling in the mornings.

The changes in steroid profiles seen after the induction

of ovulation with 4 i.u. PMSG are similar to those observed around the time of the first ovulation (Meijs-Roelofs et al., 1975; Andrews et al., 1980), or prior to ovulation in the adult rat (Bélanger et al., 1981). Similar patterns were observed in 40 i.u. PMSG-superovulated animals, although the increase in oestradiol concentrations was significantly higher, possibly due to the stimulation of a larger number of follicles; the amount of oestradiol secreted by follicles increases simultaneously with their growth (Szołtys, 1981).

No record was kept of the size of follicles on day 0 after infusion of FSH. However since the number of oocytes recovered on day 1 was approximately 30 it can be assumed that at least this number of follicles develops. Since the total ovarian oestradiol content on day 0 after infusion of FSH is only as high as that following stimulation with 4 i.u. PMSG, yet the number of ovulations is comparable to stimulation with 40 i.u. PMSG, it may be concluded that either PMSG is a far more effective stimulator of steroidogenesis than of follicular development, or that follicles stimulated by FSH do not grow as large as following stimulation with PMSG, although ovulation is induced.

Following ovulation, oestradiol levels remained elevated, in the SOV group of rats, whereas concentrations in control, FSH infused and SOV a/s rats returned to basal values similar to those observed in other studies (Watson et al., 1975; Forcelledo et al., 1981). The finding of increasing serum progesterone concentrations during the first days of pregnancy (Figures 7 and 8) is in agreement with other reports (Pepe and Rothchild, 1974; Beattie et al., 1977;

Sanyal, 1978; Forcelledo et al., 1981). Concentrations in both SOV and SOV a/s-treated rats rise to ~ 3-fold those in control animals; a similar drastic increase is seen in superovulated hamsters (Greenwald, 1976). This increase is presumably related to the increased number of corpora lutea (Miller and Armstrong, 1981a). Since the high concentrations of both ovarian and serum progesterone preclude the possibility of luteal failure, it is more probably the elevated levels of oestradiol which are responsible for the pregnancy failure in the SOV group of rats.

Results from the present study show that a/s treatment of SOV animals reduces ovarian and serum concentrations of oestradiol to values close to control animals and this reduction occurs within 18 h of a/s injection (Figures 8 and 9). This decrease in oestradiol concentrations may have been the cause of the increased embryo recovery rates in the a/s-treated group. Similarly, following ovulation in the FSH-infused animals ovarian oestradiol content was reduced to the level seen in control 4 i.u. animals (Figure 17) and embryo recovery rates in the FSH-infused rats were comparable to the SOV a/s rats in the earlier study (Figure 15).

Progesterone concentrations were unchanged by the administration of antiserum. This suggests that the presence of PMSG in the circulation is unnecessary for the continuing support of the large number of corpora lutea, originally stimulated to develop by the PMSG. Alternatively, it is possible that some PMSG may remain bound in the ovary, but only to those follicles destined to luteinize. Continued follicular stimulation does not occur, as judged by ovarian oestradiol



content on day 1 after antiserum.

The conclusions of the present study confirm the earlier work by Miller and Armstrong (1982), in which ovariectomy on day 1 of pregnancy and steroid replacement with a regimen known to maintain pregnancy allowed implantation in 50% of animals. These same authors showed that, if a very high level of oestradiol (500 ng twice daily) was administered following ovariectomy, very few animals were found to be pregnant on day 8.

Miller and Armstrong (1981b) noted that on days 0-2 of pregnancy there were large numbers of follicles in the 40 i.u. PMSG-treated rat and that the number declined after this. At the same time corpora lutea or luteinized follicles were already present on day 0 and the number increased by day 2. This suggested a continuing generation of large follicles, probably resulting from the continuing presence of PMSG in these animals, and it was suggested that these follicles secreted oestradiol which was detrimental to embryo survival. Injection of a/s may prevent development of these follicles by removal of the PMSG. On day 3 of pregnancy, a time when major losses of embryos are occurring in SOV animals, histological studies show that antiserum-treated rats exhibit a small decrease in the number of follicles  $< 100 \mu\text{m}$ , which is not significant, and a significant reduction in the number of follicles  $> 500 \mu\text{m}$  (Figure 12). These large follicles may be responsible for the high levels of oestradiol seen during the first 48 h of pregnancy in SOV animals.

Sasamoto (1962) has estimated that the circulating half-life of PMSG is  $\sim 6$  h in the mouse, whereas he concludes that its

biological life is 54-60 h (Sasamoto et al., 1972). This suggests that tissue-bound PMSG may be responsible in large part for its prolonged action. If this is the case the results of the present study suggest that the a/s was neutralizing tissue-bound PMSG in addition to any remaining in the circulation.

High circulating and ovarian oestradiol levels may be detrimental to embryonic development, since two-cell mouse embryos cultured in oviductal fluid from oestrogen-dominated donors were significantly less able to develop to morulae or blastocysts (Cline et al., 1977). However these authors suggested that oestradiol concentration may not be the only factor involved, since addition of oestradiol to culture media, in higher than physiological concentrations, had no detrimental effect, suggesting that another factor may be involved. It appears likely, from circulating oestradiol levels in the present study that PMSG stimulation results in an oestrogen-dominated oviductal fluid. Similarly, it has been shown that the influence of oestradiol in the ampulla of the ovariectomized rabbit is deleterious to normal embryo development in vivo (Stone and Hammer, 1977) and in vitro (Stone et al., 1977). In vivo the development of rat embryos to the blastocyst stage is retarded in animals which have been exposed to an extended preovulatory period of high serum oestradiol levels (Butcher and Pope, 1979). Administration of oestradiol on day 1 of pregnancy in the mature rat causes increased tubal and uterine motility, so that zygotes are expelled from the uterus within 48 h post coitus (Greenwald, 1961; Banik and Pincus, 1964; Ortiz et al., 1979). The situation in the rat is therefore different from other species in

which exogenous oestrogens may cause tube-locking (Greenwald, 1967). Forcelledo et al. (1981) have suggested that a shift in the oestrogen/progesterone ratio may also cause premature transport of the embryos to the uterus and their subsequent loss. All the above studies suggest that oestrogens may produce both detrimental oviductal and uterine environments. In the present study it is not possible to separate the effects of oestradiol, but it may be postulated that the infertility associated with superovulatory doses of PMSG is produced, at least in part, via increased ovarian oestradiol secretion.

The timing of the LH surge was not determined in the present study due to the cross reactivity of PMSG with the LH antibody. However, Armstrong and Kennedy (1972) showed that in PMSG-treated immature rats, under lighting conditions identical to the present study, the LH surge occurred between 1800 and 2000 h on the second day after injection of 4 i.u. PMSG. Sasamoto and Johki (1975) showed that, under the same lighting conditions injection of 3 i.u. PMSG into 26-day-old rats resulted in peak LH levels at 1700 h. In a similar study in 29-day-old rats on a 12 h light 12 h dark cycle Yamamoto et al. (1978) showed peak levels at 1700-1800 h. PMSG a/s will be diluted in the peripheral circulation and it is therefore unlikely to bind endogenous LH, since binding was shown to be low even at high concentrations of a/s (Chapter 5). In the present study administration of a/s at 1800 h on day 0 did not prevent ovulation, since there was no difference in the number of oocytes recovered on day 1 in a/s-treated or control animals, and the LH surge is therefore presumed to have occurred, although the exact timing is unknown.

Despite the high dose of PMSG, endogenous LH is still required for ovulation, since hypophysectomy or pharmacological blocking of the central nervous system 52 h after PMSG prevents superovulation in immature rats (Zarrow and Quinn, 1963).

In the present studies only 50% of SOV a/s rats showed evidence of implantation on day 8 which suggests that pregnancy failed in 50% of these animals between days 5 and 8. SOV animals were never found to be pregnant on day 8 (Figure 4), whereas implantation sites were found in all rats superovulated by FSH infusion (Figure 14). The major losses occurred in SOV rats prior to day 5, whereas in SOV a/s rats there were losses both prior to day 5 and also around the time of implantation. Similarly in both mice (McLaren and Michie, 1959) and hamsters (Greenwald, 1976; Fleming, 1982) after superovulation the largest prenatal losses occur early in pregnancy, prior to implantation. In the immature rat other workers (Zarrow et al., 1968) have been able to show maintenance of pregnancy in 50% of animals injected with doses of PMSG as high as 45 i.u., which suggests that there may be a possible strain difference; their study used Wistar rats.

Miller and Armstrong (1981b) observed delayed implantation in a substantial proportion of rats injected with 16 i.u. PMSG; the authors suggested this was due to a diminished uterine sensitivity to endogenous nidatory oestrogen. Similarly Yamamoto et al. (1980) recovered unimplanted embryos from the uterus on day 7 after low doses of PMSG. In the present set of experiments 50% of SOV a/s rats received exogenous nidatory oestrogen (SOV a/s  $E_2$  group), but this did

not increase the percentage of rats with implantation sites on day 8 (Figure 4). In addition, blastocysts could be flushed from only a small percentage of rats on day 7, suggesting that in most animals not showing implantation the blastocysts had been lost between days 5 and 7. Failure to implant and subsequent loss of blastocysts could result from the presence of a uterine environment unsuitable for implantation, asynchrony in embryo and uterine development or abnormalities in the blastocysts, any of which might cause expulsion or degeneration of the blastocysts, through mechanisms as yet undetermined.

Under normal circumstances the secretion of hormones by the ovary during the oestrous cycle and early pregnancy ensures that the uterine sensitivity for implantation coincides with the presence of a mature blastocyst (Psychoyos, 1973b) and, in the rat, both progesterone and oestrogen are essential for implantation. In the SOV a/s-treated rat both serum and total ovarian progesterone concentrations were higher over the first 5 days of pregnancy compared to controls (Figures 7 and 9), but Yochim and DeFeo (1963) have suggested that high concentrations of progesterone do not inhibit uterine sensitivity. Serum oestradiol concentrations in SOV a/s rats were slightly higher than in control animals between days 1-5, but total ovarian content was not significantly different (Figures 8 and 9). Courrier (1950) has suggested that the optimal conditions for embryo attachment and implantation depend on a delicately balanced synergistic action of oestrogen and progesterone on the endometrium, and this balance may have been disturbed by the different ratio of oestradiol to progesterone in SOV a/s-treated rats. Superovulation also results in

high preovulatory concentrations of serum oestradiol (Figure 9) and the possibility exists that these high levels of oestradiol, to which the uterus was exposed, may have caused uterine changes which were detrimental to blastocyst implantation 5 to 6 days later. This possibility is supported by the work of Butcher et al. (1969a) who, using egg-transfer techniques, have suggested that high preovulatory oestradiol levels independently affect oocytes and the uterus, resulting subsequently in higher embryonic mortality rates and decreased implantation rates, respectively. The contrasting implantation data in 40 i.u. PMSG + a/s vs. FSH-infused rats (Figure 19) support the idea of detrimental effects of high preovulatory oestradiol concentrations, since values were much lower in FSH-infused animals (Figure 17) and implantation occurred in 100% of these rats (Figure 14).

Implantation was probably delayed in a proportion of SOV a/s-treated rats since mean foetal weights on day 20 were considerably lower than those observed in control rats, whereas administration of nidatory oestrogen to SOV a/s rats resulted in foetuses of normal weight on day 20 (Table 5). In both rats (Hooverman et al., 1970) and mice (Enzmann et al., 1932; Enzmann, 1935) foetal development proceeds at a relatively fixed rate following implantation, and the decreased foetal weight observed on day 20 is therefore more likely to reflect a delay in implantation rather than retarded foetal growth. This delay would be similar to that observed by Miller and Armstrong (1981a) in rats injected with 16 i.u. PMSG. From the small number of rats involved it is not possible to determine if a

14  
similar delay occurred in the FSH-infused group, since implantation weights on day 8 were comparable to SOV a/s-treated animals in the earlier study, but foetal weights on day 20 were similar to control animals.

The mean foetal weights on day 20 in the present study were similar to those on day 19 in an earlier study by Stotsenburg (1915) and this 24 h difference may reflect the use of immature mothers in the present study. In mice the weight and size of the mother have been reported to have a pronounced influence on the birth weight of the young (Bluhm, 1929). In addition, there may be a small delay in implantation in all immature rats induced to ovulate, since Miller and Armstrong (1981a) found an increase in day 20 foetal weight after administration of nidatory oestrogen to control animals. The suggested delay in SOV a/s rats would therefore be a more pronounced manifestation of this observation. Koch and Oettel (1977) have shown an ~ 4 day increase in the length of gestation in immature rats, which they suggest is due to a developmental delay caused by a relative deficiency of progesterone, and not delayed implantation. These conclusions are based on measurement of the ability of the uterus to undergo decidualization; no attempt was made to flush free embryos from the uterus during the suggested period of delay. Although these authors do not report foetal weights it is probable that weights would be lower on day 20 if growth is to continue for a further 5 days prior to parturition. While a developmental delay cannot be excluded in the present studies the total delay of implantation and/or development is probably only of ~ 24 h duration.

In mice overcrowding of fetuses within the uterus has been shown to cause a decreased foetal weight (Evans et al., 1981). Foetal weight in adult rats is unaffected by the number of fetuses when this is relatively small (1-8) (Zambrana and Greenwald, 1971; Norman and Bruce, 1979). In the present study no correlations were observed in the superovulated animals, possibly because the number of animals in these groups was relatively low (Table 6). However, a negative correlation was found in control animals suggesting that the immature rat may be different from the adult in this respect. This may reflect the increased nutritional demands of pregnancy on an immature animal which is itself still growing. Placental weight was also negatively correlated with the number of fetuses, which is similar to results reported by other authors (Zambrana and Greenwald, 1971; Csapo and Wiest, 1973; Norman and Bruce, 1979).

Several authors have measured peripheral serum progesterone concentrations at various times during pregnancy in both the immature (Nutti et al., 1975; Miller and Armstrong, 1981b) and the adult rat (Csapo and Wiest, 1969; Wiest, 1970; Morishige et al., 1973; de Lauzon et al., 1974; Egg et al., 1974; Pepe and Rothchild, 1974; Elbaum et al., 1975; Ogle and Kitay, 1977; Shaikh et al., 1977; Sanyal, 1978). The results obtained on days 8 and 20 in control rats in the present study agree fairly closely with the published results. Most authors report a gradual rise in progesterone levels until ~ day 14 (Morishige et al., 1973; Egg et al., 1974; Pepe and Rothchild, 1974; Shaikh et al., 1977), with the exception of a small trough around the time of implantation (Morishige et al., 1973; Egg et al., 1974; Pepe



and Rothchild, 1974). The above authors have shown that levels plateau until days 19-21 when they decline sharply, whereas Nuti and Meyer (1975) have reported a more gradual decline from day 12 to day 20. Ovarian progesterone content has also been shown to increase up to a maximum on day 12 and then gradually decline (Wiest, 1970; Egg et al., 1974). The decline in serum progesterone concentrations at the end of pregnancy occurs even in ovariectomized rats maintained on progesterone therapy, which suggests an extraovarian mechanism in progesterone metabolism (Csapo and Wiest, 1973). Although some placental synthesis of progesterone occurs in the rat (Chan and Leatham, 1975), the corpora lutea are the major source of progesterone throughout pregnancy. Elbaum et al. (1975) have correlated serum progesterone concentrations on day 16 with total luteal weight and Kato et al. (1979) and Miller and Armstrong (1981a) have correlated it to the number of corpora lutea on days 15 and 20 respectively. The decline in progesterone production by the corpora lutea towards the end of pregnancy and the lower concentrations of serum progesterone in the SOV a/s  $E_2$ -treated rats, compared to SOV a/s rats (Table 9) may indicate that the oestrogen-treated animals were closer to the end of pregnancy than the SOV a/s rats, in which it is postulated that implantation was delayed and gestation therefore lengthened.

In the present study on day 20 serum and ovarian progesterone levels in SOV a/s and SOV a/s  $E_2$  rats are 2- to 4-fold higher than concentrations in control rats (Table 9), which probably reflects increased production by the larger number of corpora lutea in superovulated rats. Miller and Armstrong (1981a) found a good

correlation between the number of corpora lutea and serum progesterone concentrations on day 20 in rats receiving 4, 8, or 16 i.u. PMSG. However, ovarian progesterone content in FSH-infused rats on day 20 (Table 11) was similar to control animals in the previous study, which suggests that either the corpora lutea in these animals are not producing as much progesterone, or that some corpora lutea failed earlier in the pregnancy. It has been shown that partial lutectomy has no effect on the maintenance of pregnancy (Nutti and Meyer, 1975), suggesting that excess progesterone is normally present and in the FSH-infused group an early regression of some corpora lutea, with no effect on pregnancy, cannot be excluded.

The effects of high concentrations of progesterone on embryo development cannot be determined in these studies, since there are many possible causes of increased foetal mortality in superovulated rats. However several authors have injected high doses (up to 16 mg) of progesterone daily throughout pregnancy and have reported no effect on the length of gestation or the viability of the foetuses (Sammelwitz et al., 1956; Kraicer et al., 1971), although Bartholomeusz and Bruce (1976) injected daily 5 times the normal endogenous 24 h production and found an increase in the number of dead foetuses, most of which had died prior to day 14.

Serum oestradiol concentrations in control animals were slightly lower than reported by Miller and Armstrong (1981a) in immature rats on day 20 or by de Lauzon et al. (1974) for adult animals on days 8 and 20. However, the number of foetuses in the present study was also lower and it has been suggested that ovarian

oestrogen secretion is regulated in part by the number of live fetuses (Csapo and Wiest, 1973; Crosskerry and Dobbing, 1978; Kato et al., 1979).

In the normal adult rat losses prior to and during implantation are higher than those occurring post implantation and the majority of postimplantation losses occur prior to day 11 (Harper, 1964), which is similar to the situation observed in the present study, particularly in SOV a/s rats. The majority of embryos which implant in these animals continue to term, although there is a small loss between days 8 and 20. However, the major loss of embryos in SOV a/s-treated rats occurs between days 5 and 8 when pregnancy fails in ~ 50% of rats. The possibility exists that this failure may be the result of failure of the uterus to provide a suitable environment for implantation, or that blastocysts recovered on day 5 from SOV a/s rats may be unable to continue to develop, even if the environment were normal.

Although when using infusion of FSH it was not possible to produce superovulation in all animals, when superovulation was produced the pregnancy was generally maintained (Figure 14), unlike the situation in SOV a/s-treated rats where less than 40% of rats maintained their pregnancy. The improvement may be related to the short half-life of FSH, which has been calculated to be ~ 90 min in the rat (Laster, 1972) compared to the half-life of PMSG which has been estimated to be as long as ~ 26 h (Parlow and Ward, 1961). Thus removal of the infusion pump will result in a rapid decline in circulating FSH and contaminating LH and prevent continued stimulation

of the follicles. The rapid decline in ovarian oestradiol content (Figure 16) suggests that this does indeed occur.

From the studies presented in this chapter it may be concluded that a large number of oocytes can be recovered from immature rats by superovulation with either a single injection of 40 i.u. PMSG or continuous infusion of FSH, and the infertility associated with the former is probably produced, at least in part, via increased ovarian oestradiol secretion. Use of a PMSG a/s has shown that the increased oestradiol secretion is a result of continuous ovarian stimulation, as opposed to continued secretion by a previously hyperstimulated ovary. However, use of this antiserum cannot correct all the abnormalities caused by the PMSG and pregnancy only continues to day 20 in ~ 40% of SOV a/s rats; the major loss of pregnancy occurs in these animals around the time of implantation.

CHAPTER 7  
PREIMPLANTATION STAGES OF PREGNANCY  
FOLLOWING SUPEROVULATION

7.1 Introduction.

Superovulation with exogenous gonadotrophins has become a well-established technique for increasing the supply of embryos, but it may result in reduced fertility in both large domestic animals and in small laboratory animals (see Chapter 2). Administration of these hormones may not exactly mimic the normal endocrinological background for oocyte maturation and ovulation and it has been suggested that the procedure may result in oocyte abnormalities. In particular, atretic follicles may be 'rescued' and contribute defective oocytes along with 'normal' ones (Bavister, 1982). The yield of viable embryos may be highly variable and retarded or abnormal embryos are often recovered (Betteridge, 1977). Under such circumstances it is difficult to establish whether the primary defect occurred before or after the time of ovulation. Studies in which superovulated immature rats were ovariectomized at different intervals after the time of mating suggest that at least a large proportion of the embryos present during the first 36 h after the time of mating are normal, and that the failure to develop to the blastocyst stage in superovulated

rats results from an abnormal ovarian hormone secretion after ovulation, rather than abnormalities in the oocyte per se (Miller and Armstrong, 1982). Other authors have suggested that, following superovulation, a small proportion of oocytes are degenerate at the time of ovulation (Sherman et al., 1982).

Since a large number of oocytes can be recovered from the oviducts of superovulated immature rats on day 1 of pregnancy the ovulatory process is probably not defective (Austin, 1950). Between days 1 and 2 there is a substantial reduction in the number of oocytes and/or embryos recovered from the reproductive tracts of these animals (Chapter 6), although this reduction may be partially accounted for by the loss of degenerate oocytes, ovulated 24 h earlier than the normal time of ovulation (Miller and Armstrong, 1981b). Gonadotrophic stimulation has been shown to result in the ovulation of at least two sets of oocytes in both mice (Fowler and Edwards, 1957; Stern and Schuetz, 1970; Kaufman and Whittingham, 1972) and rats (Zarrow and Gallo, 1969; De la Lastra et al., 1972; Park and Zarrow, 1972; De la Lastra and Forcelledo, 1973; Kostyk et al., 1978; Miller and Armstrong, 1981b). Administration of oestradiol at the time of PMSG injection (Park and Zarrow, 1972) and of progesterone, 24 h after PMSG (Ying and Meyer, 1969b; Zarrow and Gallo, 1969) enhances this effect, both in terms of the number of rats ovulating and the number of oocytes shed. In farm livestock the length of time over which multiple ovulations are spread after superovulation is unknown, but it has generally been assumed to be long enough to necessitate repeated inseminations at approximately 12 h intervals during and

immediately after oestrus, to ensure good fertilization rates (Betteridge, 1977).

In the research described in this chapter various stages of preimplantation development were studied, in an attempt to determine what contribution each might make to the infertility following superovulation. The timing of ovulation was investigated since asynchrony between ovulation and mating could result in reduced fertilization rates. The normality of oocytes recovered from superovulated donors was assessed using the technique of transfer of the oocytes to a normal adult recipient and subsequent fertilization and foetal development in the recipient. An additional study was designed to investigate whether fertilization failure was a cause of reduced fertility in superovulated rats, as was suggested by a previous study (Austin, 1950). Earlier work (Chapter 6) had suggested that superovulation resulted in expulsion of embryos from the reproductive tract, since embryo recovery decreased with time after ovulation in superovulated rats and embryos were never recovered predominantly from the uterus. Concentrations of ovarian and serum oestradiol are high in superovulated animals (Chapter 6) and several authors have shown that administration of exogenous oestrogens to rats results in premature passage of the embryos from the oviducts to the uterus and subsequent expulsion (Greenwald, 1961; Banik and Pincus, 1964; Ortiz et al., 1979). If the effect of oestradiol is just accelerated transport then it might be possible, by the use of ligatures, to retain the embryos in the oviducts until day 4 when they would normally be passing into the uterus (Psychoyos, 1967). In addition, a specific anti-oestradiol serum and pharmacological blockers

of oestradiol synthesis were used to reduce circulating levels of oestradiol in superovulated rats.

## 7.2 Timing of Ovulation

### 7.2.1 Methods

In this experiment the rats were not mated. Immature rats received 4 or 40 i.u. PMSG on day -2 and animals in the control group were sacrificed at 1 h intervals, from 2330 h on day 0 until 0530 h on day 1. A further group was sacrificed at 0900 h on day 1. The number of ovulation points on each ovary was counted. Superovulated rats were sacrificed at 3- or 6 h intervals from 1800 h on day -2 until 0900 h on day 1. Since the large number of ovulations made it impossible to obtain accurate counts of ovulation points on the ovary the oviducts were flushed and recovered oocytes were counted. Rats from which no oocytes were recovered were included in the calculation of means. From 1800 h on day 0 to 0900 h on day 1 the number of freshly ovulated oocytes was estimated from the number of distinct masses of cumulus. Since one-cell oocytes, with and without cumulus, and fragmented oocytes tended to become clumped in the dish, the flushings were then exposed to 0.1% hyaluronidase for 5 min and the total number of oocytes, together with the number of fragmented oocytes, were recorded. The number of one-cell oocytes without cumulus could be estimated by subtraction of the number of distinct masses of cumulus from the total number of one-cell oocytes. Oocyte recovery over time was analyzed



by linear regression, after arbitrary division into 24-h time periods (see Results) and the slopes of the lines were compared to zero (Steel and Torrie, 1960). The effects of time on the total number of oocytes, and numbers of fragmented oocytes or freshly ovulated cumulus masses were compared using one-way analysis of variance, and differences between times were assessed using Duncan's New Multiple Range Test.

### 7.2.2 Results

Figure 21 (lower panel) shows that control rats ovulated between 0030 and 0530 h on day 1. By 0530 h 100% of large follicles visible on the ovary had ovulated and there was no statistical difference in the number of ovulations from 0330 h to 0900 h.

Superovulated animals showed a more complex pattern of ovulations. Figure 21 (upper panel) shows the percentage of rats from which oocytes were recovered at any particular time. With the exception of one rat which had ovulated by 1800 h on day -2, no ovulations were observed prior to 0600 h on day -1. The majority of animals had ovulated by 1500 h on day -1, although as late as 1800 h on day 0 no oocytes were recovered from 2/12 rats sacrificed.

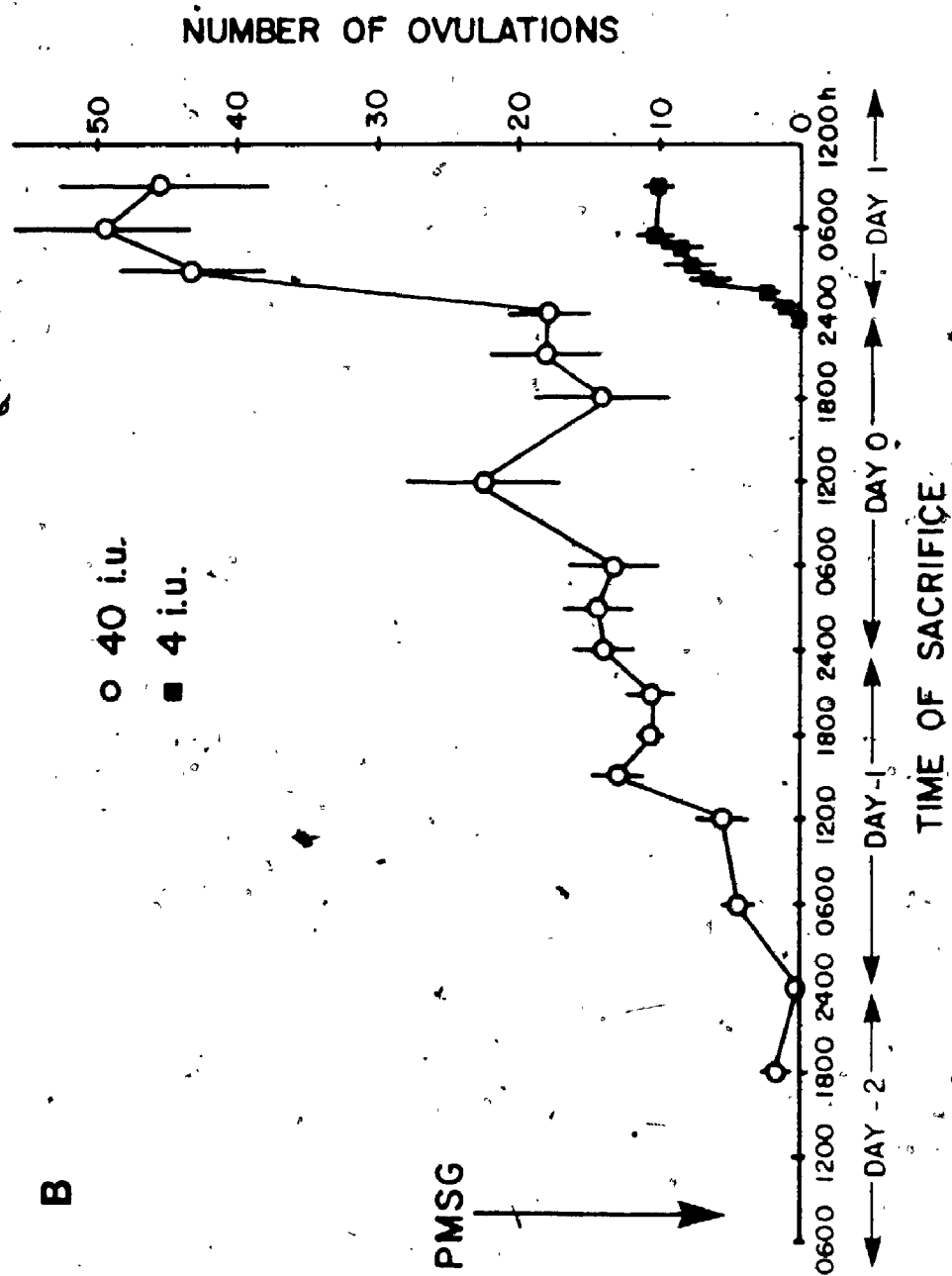
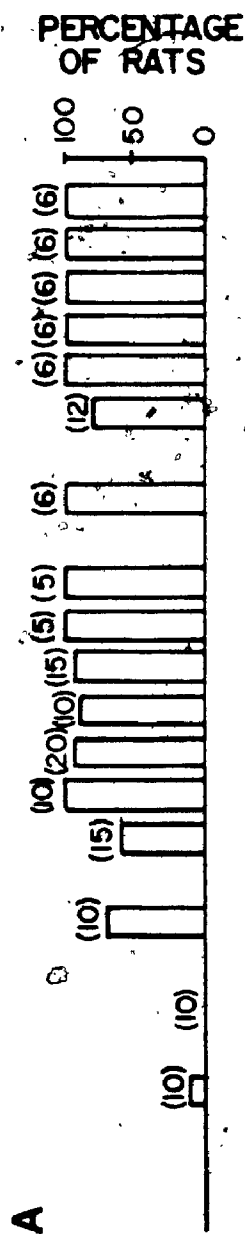
Figure 21 (lower panel) shows that the number of oocytes recovered from the oviducts of these superovulated rats increased significantly ( $P < 0.01$ ) throughout day -1 with no significant increase throughout day 0. The regression of oocyte recovery rate on time was described by  $Y = (0.52 \pm 0.11)X + 1.66$  and  $Y = (0.22 \pm 0.12)X + 14.16$  for day -1 and day 0, respectively, where  $Y$  = oocyte recovery rate and  $X$  = time. With the exception of one superovulated animal which had two fragmented

FIGURE 29

Timing of ovulation in immature rats induced to ovulate with 4 or 40 i.u. PMSG.

A. Percentage of rats displaying ovulation after 40 i.u. PMSG (Numbers indicate the numbers of rats sacrificed at each time).

B. Number of ovulations per rat after treatment with 4 i.u. ( $n = 4$  rats per point) or 40 i.u. PMSG ( $n = 5 - 20$  rats per point, as in A). Values are mean  $\pm$  S.E.M. Rats from which no oocytes were recovered are included in the calculation of means.



oocytes in the oviduct at 0300 h on day 0 no fragmented oocytes were recovered prior to 1800 h on day 0. However animals were occasionally seen where there was very little or no cumulus mass surrounding the oocytes. Figure 22 shows a more detailed breakdown of the oocytes recovered 58-73 h after PMSG in superovulated rats. The number of oocytes recovered varied considerably between animals, but there was a significant ( $P < 0.001$ ) increase in both total oocytes recovered and the number of cumulus masses from 2400 h on day 0 to 0600 h on day 1. From then onwards there was no significant increase. The number of fragmented oocytes did not increase significantly over this 15 h period.

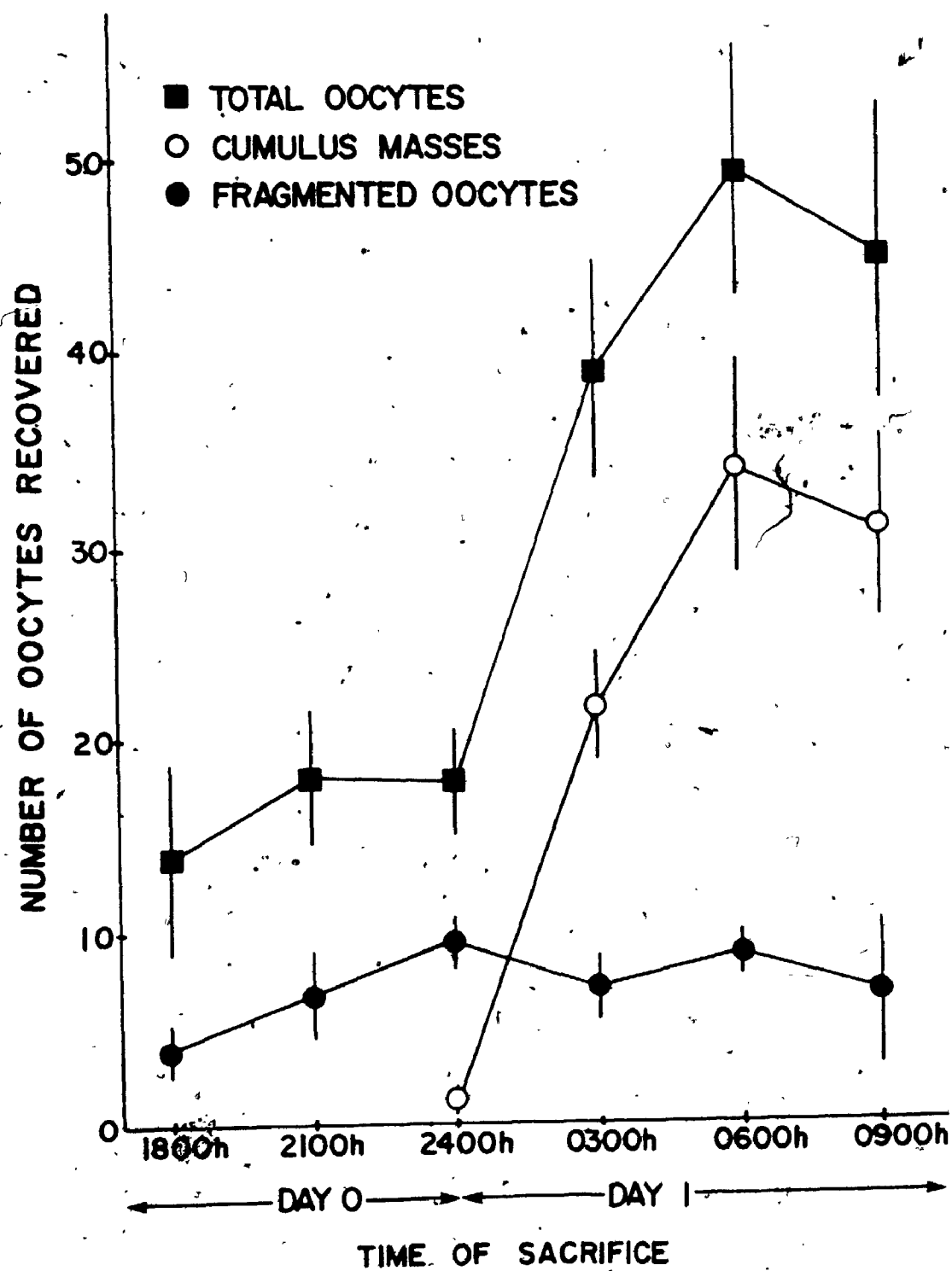
### 7.3 Normality of Oocytes

#### 7.3.1 Methods

Oocytes from albino Sprague-Dawley (S-D) rats were transferred to pigmented Long-Evans (L-E) rats so that endogenous and transferred foetuses could be distinguished. Immature female S-D rats and mature female L-E rats were provided with lighting for 14 h daily, but the timing of the period of illumination of the two groups of rats was altered to manipulate the time of ovulation. Adult animals were allowed at least 2 weeks to adjust to the change in their lighting schedule. Immature animals were housed in altered lighting from their arrival (45-50 g) until sacrifice. All times cited in the text refer to the animals' diurnal cycle; thus 0000 h refers to the midpoint of the animals' dark period and 1200 h is the midpoint of the light period. The lighting of the immature S-D rats

FIGURE 22

Recovery of cumulus masses and fragmented oocytes from the oviducts of immature rats 58 - 73 h after induction of ovulation with 40 i.u. PMSG. Values are mean  $\pm$  S.E.M. (Number of rats as in figure 21(A)).



was advanced 2 h relative to the adult L-E animals.

The immature S-D donor rats were injected with 4 i.u. PMSG (control) or superovulated with 40 i.u. PMSG at 0830 h on day -2.

Between 0300 h and 0600 h on day 1 the donors were sacrificed and the oviducts were flushed as described previously (Chapter 6) using sterile DBS + 5% rat serum (heat-inactivated, charcoal-treated).

Recovered oocytes were classified as degenerate, 1-cell with cumulus and 1-cell without cumulus. Only 1-cell oocytes were transferred.

Follicular oocytes were recovered from 4 i.u. or 40 i.u. donors where large unovulated follicles were visible on the ovaries. Follicular oocytes were recovered by puncturing large follicles with a 30-gauge needle and expressing the oocyte. Two to four oocytes from either a control or a superovulated donor were transferred to the bursa of a recipient (see below) by means of a heat-polished glass pipette attached to a mouth tube. In any one transfer all the oocytes either had cumulus or were naked. Where a large number of oocytes was recovered from a particular donor, oocytes were selected for transfer at random. A maximum of three transfers was done with oocytes from any one donor. Both follicular and oviductal oocytes were collected from some donors, but any one recipient received only follicular or oviductal oocytes.

Figure 23 shows a schematic representation of the procedure used for the bursal transfers. The adult recipient L-E rats were synchronized using a modification of the method described previously (Vickery and McRae, 1980). Fifty µg of an LHRH agonist [Des-Gly<sup>10</sup>, D-Ala<sup>6</sup>, Pro-NHET<sup>9</sup>] LHRH

FIGURE 23

Schematic representation of the procedure used for bursal transfers. Light and dark areas indicate periods of light and darkness respectively. The numbers refer to the day of the experiment (where day 1 = first day of pregnancy).



4 IU or 40 IU  
PMSG s.c.

SACRIFICE

DONORS -4 -3 -2 -1 0 1

2 hr shift  
in light cycle

RECIPIENTS

-4 -3 -2 -1 0 1 2 20

SYNCHRONIZE  
WITH LHRH

MATE

SACRIFICE:  
FOETAL & PLACENTAL  
WEIGHTS

SCORE FOR MATING  
& TRANSFER

were injected s.c. at 0600 h on day -4 and animals were caged with males during the evening of day 0. At 0030-0100 h on the morning of day 1 the rats were scored for mating, using the presence of a copulatory plug and/or spermatozoa in the vagina as evidence, and only mated rats were used in the subsequent transfer. Transfers were performed between 0100 h and 0400 h.

Recipient L-E rats were anaesthetized with tribromoethanol solution (2%, 0.01 mg/g body weight) and transfers were carried out using procedures similar to those described by Noyes (1952). One ovary was exposed through a flank incision and, holding only the surrounding adipose tissue, the ovary was drawn out, care being taken to avoid handling either the ovary or the reproductive tract. The transfer pipette was passed through the foramen in the bursal membrane (Alden, 1942a) and the oocytes were expelled beneath the bursal membrane. The ovary was replaced in the abdomen and the wound was closed. Transfers were made only to one side of any rat.

Recipient rats were sacrificed on day 20 and the total number of foetuses together with the number of live foetuses was recorded in both the control (nontransferred) and transfer horns. In the transfer horn the foetuses were divided into pigmented (endogenous) and albino (transferred). Foetal and placental weights were recorded for all three groups, i.e., control, transfer horn pigmented and transfer horn albino groups. Any rat not pregnant in the control horn was excluded from the results.

Recipients were classified according to PMSG dose injected into the donor and the presence or absence of cumulus surrounding the

transferred oocytes. Comparisons were made within the class between the control horn and the transfer horn and between the pigmented and albino groups in the transfer horn. Mean foetal and placental weights in each group were compared using a t-test. The proportion of oocytes recovered as fetuses was compared using a  $\chi^2$  test.

In order to test the viability of those oocytes recovered earlier than 66.5-69.5 h from rats superovulated with 40 i.u. PMSG (Miller and Armstrong, 1981b), an additional study was carried out in which 32 oocytes were collected from the oviducts of 5 rats injected with 40 i.u. PMSG 26.5-29.5 h previously, and 20 oocytes were collected from the oviducts of 5 rats injected with 40 i.u. PMSG 42.5-45.5 h previously. All oocytes in these two groups were surrounded by cumulus cells and transfers were made to 6 and 9 recipient rats, respectively.

To determine if cumulus cells were essential for oocyte pick-up a small study was undertaken in which oocytes, recovered from the oviducts of 4 i.u. donors, were denuded with 0.1% hyaluronidase and then transferred beneath the bursa of a synchronized adult L-E rat as described above. Fourteen denuded oocytes were transferred to 5 recipients and 16 intact oocytes to a further 5 recipients.

### 7.3.2 Results

Following synchronization of oestrus 75-94% of recipient rats mated. The number of surviving fetuses following transfer of oocytes on day 1 is given in Table 12. The proportion of oocytes surviving following transfer from the oviducts of 4 i.u. or 40 i.u.

TABLE 12

Number of Foetuses Surviving on Day 20 following Transfer of Oocytes on Day 1  
(for further detail of transfer see text)

Dose of PMSG	Donor		Proportion of Recipients with at Least One Live Foetus	Number of Oocytes Transferred	Number of Surviving Foetuses
	Source of Oocytes	Cumulus (+/-)			
4 i.u.	oviducts	+	37/56	218	85 (39.0%)
	oviducts	+	0/4	13	0
	follicles	+	17/17	62	35 (56.5%)
40 i.u.	oviducts	+	60/78	299	113 (37.8%)
	oviducts	-	0/11	41	0
	follicles	+	14/20	75	28 (37.3%)

rats was not significantly different. Similarly, the proportion of oocytes surviving after recovery from either the oviducts or follicles of 40 i.u. rats was not significantly different. The proportion of oocytes surviving following recovery from the follicles of 4 i.u. rats was significantly higher than following recovery either from the oviducts of 4 i.u. rats ( $P < 0.05$ ) or from the follicles of 40 i.u. rats ( $P < 0.05$ ). No oocyte lacking cumulus cells at the time of transfer developed, irrespective of the dose of PMSG.

There were no significant differences in the mean foetal and placental weights between control and transfer horns. In the transfer horn there were no significant differences in the mean foetal and placental weights between endogenous pigmented and transferred albino groups (Table 13).

In the recipient rats which received oocytes collected 26.5-29.5 h after PMSG, 6/32 oocytes survived (18.8%) which was not significantly different from the percentage survival of oocytes collected from 40 i.u. rats 66.5-69.5 h after PMSG in the main trial (37.8%). However only 2/20 oocytes collected 42.5-45.5 h after 40 i.u. PMSG survived to foetuses, which is a significantly lower proportion ( $P < 0.05$ ) than the main trial. Again there were no significant differences in mean foetal and placental weights between horns or within the transfer horn.

In the recipient rats which received oocytes denuded with hyaluronidase live foetuses developed from 2/14 oocytes (14.3%) compared with 7/16 oocytes (43.8%) when cumulus was not removed. These differences are not significant, possibly due to the small numbers involved.

TABLE 13

Foetal and Placental Weights in the Transfer Horn on Day 20, after Oocyte Transfer on Day 1

Dose of		Doñor		Foetal Weight (g)		Placental Weight (mg)	
PMSG	Source of	Oocytes		Endogenous	Transferred	Endogenous	Transferred
4 i.u.	oviduct			1.98 ± 0.03 (55)	1.94 ± 0.03 (37)	437 ± 13 (55)	412 ± 9 (37)
	follicle			1.93 ± 0.04 (17)	1.83 ± 0.04 (17)	410 ± 12 (17)	397 ± 22 (17)
40 i.u.	oviduct			1.97 ± 0.03 (78)	1.97 ± 0.04 (60)	432 ± 9 (78)	427 ± 10 (60)
	follicle			1.97 ± 0.05 (20)	1.95 ± 0.05 (14)	455 ± 14 (20)	501 ± 31 (14)

Values are mean ± S.E.M. for the number of observations in parentheses.

## 7.4 Fertilization

### 7.4.1 Methods

Immature rats were injected with 4 or 40 i.u. PMSG on day -2. Following mating overnight of day 0, the rats were sacrificed at 1400 h to 1600 h on day 1 and the oviducts were flushed with DBS as described previously (Chapter 6). Oocytes recovered were counted and classified as normal or degenerate; fragmented oocytes or oocytes with more than one cell were considered degenerate. One-cell oocytes were mounted on a slide with a coverslip suspended at the corners by four spots of vaseline (Chang, 1952) and examined without staining, under a phase contrast microscope, for evidence of sperm penetration and pronuclear formation. Statistical comparisons between control and superovulated groups were made using a  $\chi^2$  test with Yates' correction.

### 7.4.2 Results

The results from 14 control 4 i.u. and 19 superovulated 40 i.u. rats are presented in Table 14. From the control rats 132 oocytes were recovered and 98.5% of these were at the 1-cell stage. A significantly lower percentage (71.6%,  $P < 0.001$ ) of the 631 oocytes recovered from superovulated rats were at the 1-cell stage; the remaining 28.4% of the oocytes appeared degenerate. In the superovulated group 69.7% of 1-cell oocytes had sperm within the vitellus and a significantly lower number (62.7%,  $P < 0.01$ ) had pronuclei. All oocytes classified as fertilized from control rats had

TABLE 14

Oocyte Recovery Rates, Fertilization Rates and Incidence of Polyspermy  
in Immature Rats Induced to Ovulate with 4 or 40 i.u. PMSG

	<u>Number of Oocytes (%)</u>		<u>Significance**</u>
	<u>4 i.u. PMSG</u>	<u>40 i.u. PMSG</u>	
Recovered	132	631	
Degenerate	2 (1.5)*	179 (28.4)*	P < 0.001
One-cell	130 (98.5)*	452 (71.6)*	P < 0.001
One-cell examined	112	373	
One-cell with pronuclei	111 (99.1) <sup>†</sup>	234 (62.7) <sup>†</sup>	P < 0.001
One-cell with sperm tail in vitellus	111 (99.1) <sup>†</sup>	260 (69.7) <sup>†</sup>	P < 0.001
One-cell with polyspermy	4 (3.6) <sup>†</sup>	12 (4.6) <sup>†</sup>	N.S.

\* % of recovered eggs.

<sup>†</sup>% of one-cell eggs examined.

\*\* P value for difference between percentages.



both evidence of sperm penetration and pronuclear formation. Any cumulus cells attached to oocytes from control rats generally dispersed during the handling and mounting procedure, whereas oocytes from most, but not all, superovulated rats, had cumulus cells firmly attached after mounting. The percentage of 1-cell oocytes fertilized was significantly higher ( $P < 0.001$ ) in control rats compared to the superovulated group (99.1% vs. 69.7%). The incidence of polyspermy was less than 5% and was not significantly different between groups. All mated rats in the control group displayed a high proportion of oocytes fertilized. Despite evidence of mating, no fertilized oocytes were recovered from 4 rats in the superovulated group and one further rat had only one fertilized oocyte of 26 ovulated. Exclusion of these 5 rats from the superovulated group still resulted in a significantly lower percentage of 1-cell oocytes fertilized than in the control group (89.3% vs. 99.1%,  $P < 0.01$ ).

## 7.5 Retention of Embryos within the Oviducts

### 7.5.1 Methods

Immature rats were injected with 4 or 40 i.u. PMSG on day -2 and caged with males from the evening of day 0. In the afternoon of day 1 the rats were anaesthetized with ether and the left ovary was exposed via a flank incision. With minimal trauma to the reproductive tract either a single ligature was tied at the uterotubular junction or the

tract was ligated both at the uterotubular junction and as close to the infundibulum as possible. The right oviduct was not ligated and served as a control, but no sham operations were performed. The animals were sacrificed on the morning of day 4 and the oviducts were flushed as described previously (Chapter 6). The normality of the recovered embryos was assessed under both light and phase contrast microscopes; embryos with irregularly shaped or sized cells were classified as abnormal. Differences between doses of PMSG and between oviducts, with respect to the percentage of rats from which embryos were recovered and the percentage of normal embryos, were compared using a 2 x 2 factorial analysis of variance after arcsin transformation of the data. Within treatments, differences were compared using a  $\chi^2$  test. Differences between the mean embryo recovery rates in control and ligated horns were compared using a paired t-test.

#### 7.5.2 Results

The percentage of rats from which embryos were recovered from each oviduct is shown in Figure 24. Overall, embryos were recovered from a significantly higher proportion of ligated oviducts compared with control oviducts ( $P < 0.01$ ), and from a significantly higher proportion of 4 i.u. oviducts ( $P < 0.01$ ) compared to 40 i.u. oviducts. There was no significant difference between the two horns in 4 i.u. rats, but embryos were recovered from a significantly higher proportion of ligated oviducts from rats treated with 40 i.u. PMSG ( $P < 0.001$ ).

When the embryos were examined (Figure 25) it was found that in both 4 and 40 i.u. rats the percentage of normal embryos in

FIGURE 24

Percentage of rats from which embryos were recovered in each oviduct after unilateral oviductal ligation (4 i.u. n = 20; 40 i.u. n = 25).

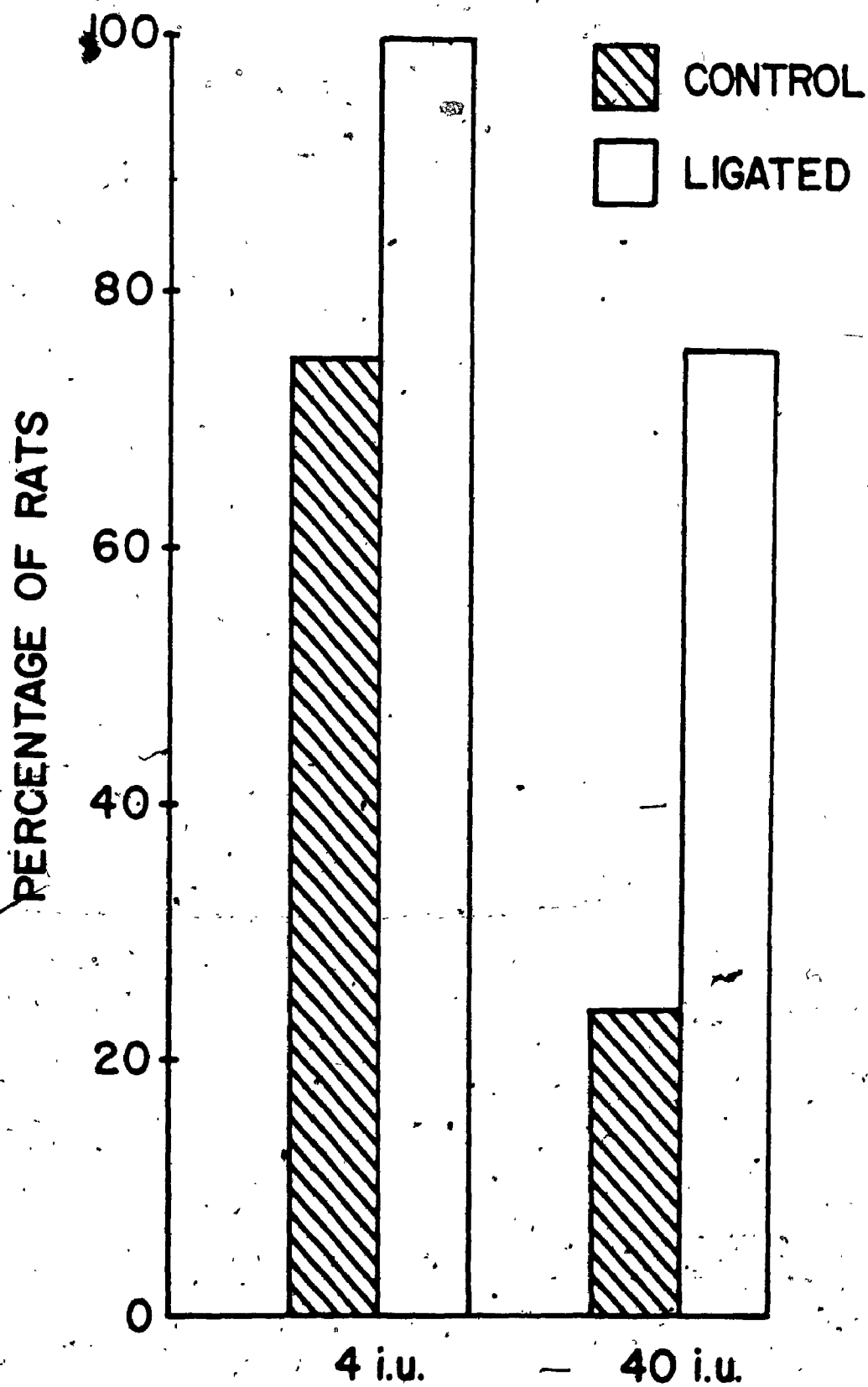
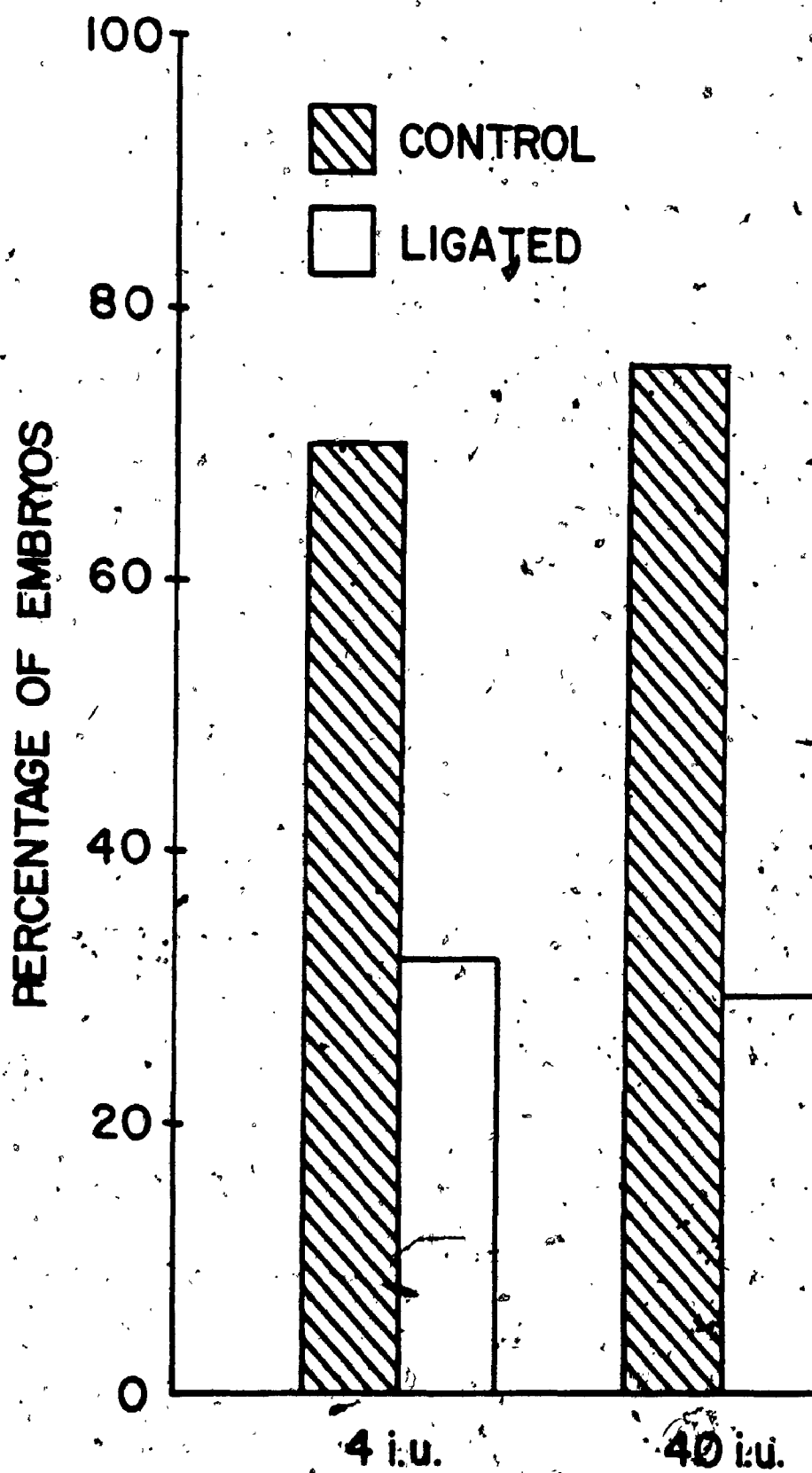
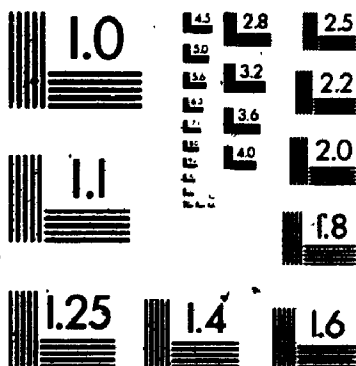


FIGURE 25

Percentage of embryos recovered, assessed as normal, after  
unilateral oviductal ligation (4 i.u. n = 20; 40 i.u. n = 25).



3



1

the ligated horn was reduced ( $P < 0.01$ ), but there was no significant difference between the doses of PMSG. Comparing rats that had a single ligature with those with a double ligature (Figure 26), a significantly higher proportion of rats with a single ligature had embryos ( $P < 0.01$ ) and the percentage of normal embryos (Figure 27) was also significantly higher ( $P < 0.01$ ). In neither comparison was there a significant difference between dose of PMSG.

The mean number of embryos recovered (Table 15) was not significantly different between control and ligated horns, except in 40 i.u.-treated rats with a single ligature where embryo recovery rate was increased significantly ( $P < 0.001$ ).

## 7.6 Role of Oestrogen

### 7.6.1 Methods

Three groups of rats were induced to ovulate with 4 i.u. PMSG and 5 groups with 40 i.u. On day 1 following mating the groups were treated with no further drugs (4 i.u. control and 40 i.u. control), a s.c. implant of 100 mg 4-acetoxy-4-androstene-3,17-dione (4 i.u. + 4AA and 40 i.u. + 4AA) as described by Brodie *et al.* (1978) or a twice-daily s.c. injection of 10 mg aminoglutethimide phosphate in saline (4 i.u. + AGP and 40 i.u. + AGP). These doses of 4AA and AGP have previously been shown to inhibit oestradiol synthesis *in vivo* in rats (4AA; Brodie *et al.*, 1978; AGP, Armstrong *et al.*, 1976). The two remaining groups of superovulated 40 i.u. rats were either injected twice daily with AGP and 2 mg progesterone, both s.c., (40 i.u. + AGP +  $P_4$ ) or twice daily i.p. with 0.4 ml of the specific



FIGURE 26

Percentage of rats with single or double oviductal ligatures from which embryos were recovered. (Numbers in brackets are the number of rats in each group).

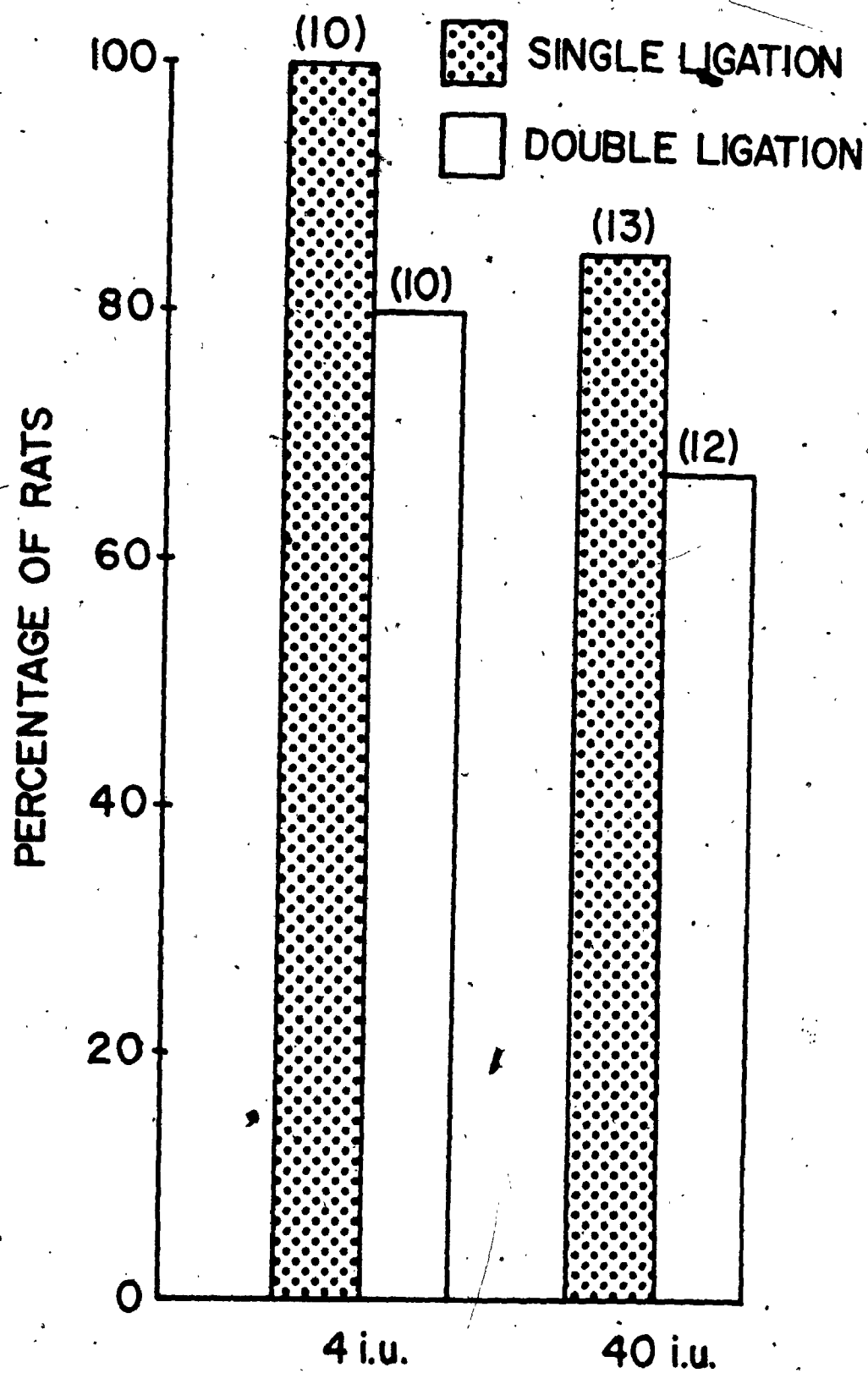


FIGURE 27

Percentage of embryos recovered, assessed as normal, from oviducts with single or double ligatures. (Numbers of rats as in Figure 26).

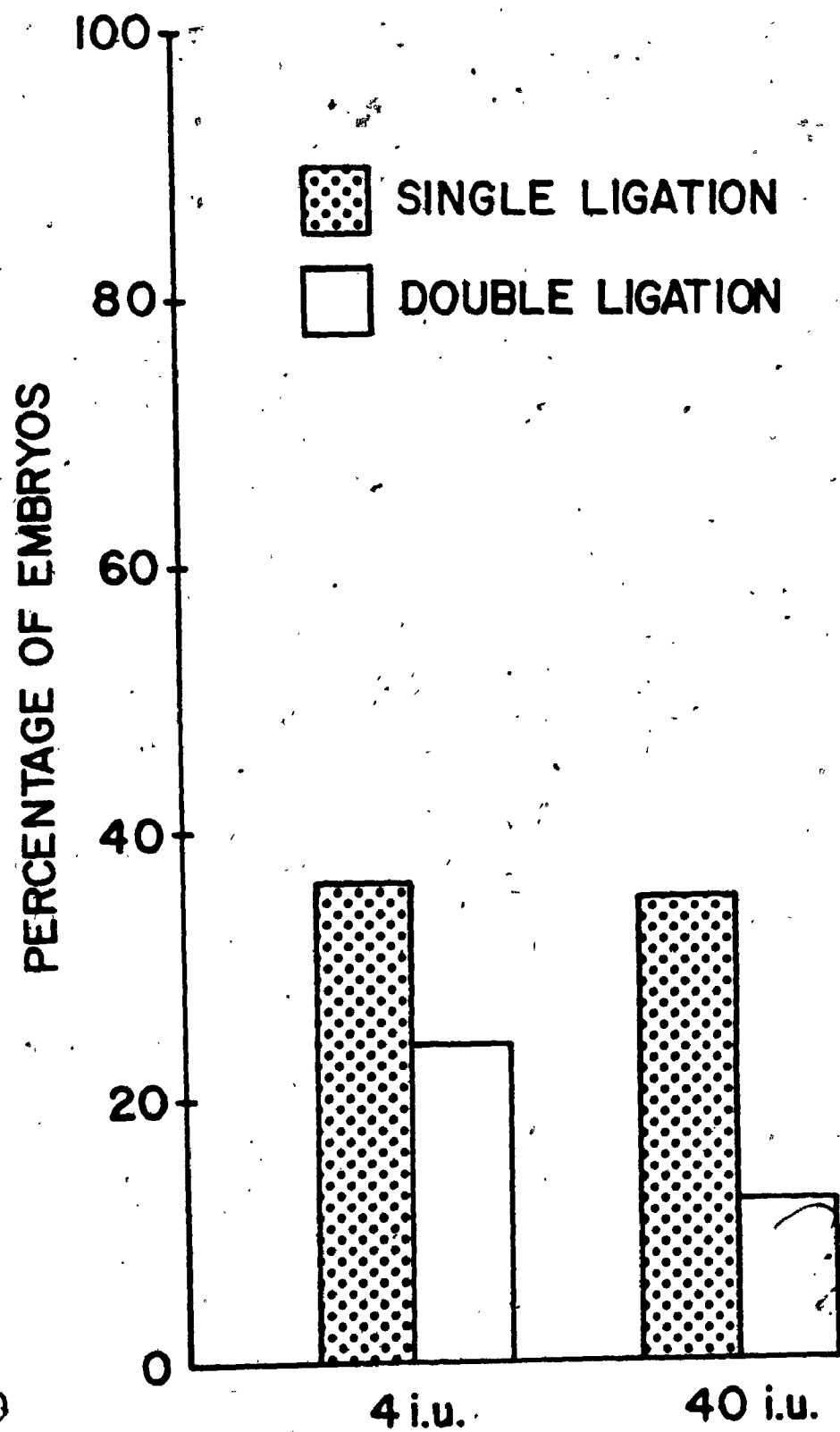


TABLE 15

Embryo Recovery Rates on Day 4 of Pregnancy after Ligation of the  
Oviducts on Day 1 in 4 i.u. or 40 i.u. PMSG-Treated Rats  
(mean  $\pm$  S.E.M.; for further details see text)

Oviduct	PMSG Treatment	
	4 i.u. <sup>1</sup>	40 i.u. <sup>2</sup>
Single Ligature:		
Control	2.8 $\pm$ 0.7	0.8 $\pm$ 0.5
Ligated	3.3 $\pm$ 0.5	8.7 $\pm$ 1.8*
Double Ligature:		
Control	2.0 $\pm$ 0.8	0.9 $\pm$ 0.6
Ligated	1.7 $\pm$ 0.3	2.8 $\pm$ 1.4

\* significantly different ( $P < 0.001$ ) from control oviduct.

<sup>1</sup>n = 10 rats per ligature group

<sup>2</sup>n = 12 rats per ligature group

anti-oestradiol serum described in Chapter 6 (40 i.u. + E<sub>2</sub> a/s). The treatments are summarized in Table 16.

All animals were sacrificed on the morning of day 3 and the oviducts and uteri were flushed to recover embryos, as described in Chapter 6. Ovarian and uterine weights were recorded and ovaries were collected for steroid analyses as described previously (Chapter 6). Serum from 40 i.u. + E<sub>2</sub> a/s and control rats was incubated overnight at 4°C in the presence of tritiated oestradiol. Bound and free hormones were separated using dextran-coated charcoal (Kushinsky and Anderson, 1975).

Differences between percentages of rats in which embryos were found, after the various treatments, were tested using a  $\chi^2$  test and differences between the number of embryos recovered, ovarian and uterine weight and ovarian oestradiol and progesterone concentrations were compared using one-way analysis of variance and Duncan's New Multiple Range Test (Steel and Torrie, 1960). Heterogeneity of variance, when present, was removed by logarithmic transformation of the data prior to analysis.

In a subsequent experiment, using only rats injected with 4 i.u. PMSG and inhibitors of oestradiol synthesis, the normality of the embryos recovered on day 3 was assessed.

#### 7.6.2 Results

The percentage of rats in each treatment group from which embryos were recovered on day 3 is shown in Figure 28. All 4 i.u. rats had embryos, whereas the percentage of superovulated rats from which embryos were recovered varied between 50% and 86%. There was no significant difference between the percentage of rats with embryos in any of the 40 i.u. + inhibitor groups. Figure 29 shows the mean

TABLE 16

## Summary of Treatments Used to Reduce Oestradiol Concentrations

<u>Group</u>	<u>PMSG</u>	<u>Further Treatment</u>
4 i.u. Control	4 i.u.	None
4 i.u. + 4AA	4 i.u.	100 mg s.c. implant 4AA
4 i.u. + AGP	4 i.u.	s.c. injection 10 mg AGP twice daily
40 i.u. Control	40 i.u.	None
40 i.u. + 4AA	40 i.u.	100 mg s.c. implant 4AA
40 i.u. + AGP	40 i.u.	s.c. injection 10 mg AGP twice daily
40 i.u. + AGP + P <sub>4</sub>	40 i.u.	s.c. injection 10 mg AGP + 2 mg progesterone twice daily
40 i.u. + E <sub>2</sub> a/s	40 i.u.	i.p. injection 0.4 ml oestradiol antiserum twice daily

FIGURE 28

Percentage of rats with embryos on day 3 after anti-oestrogen treatment.  
(For details of treatments and abbreviations see Table 16).



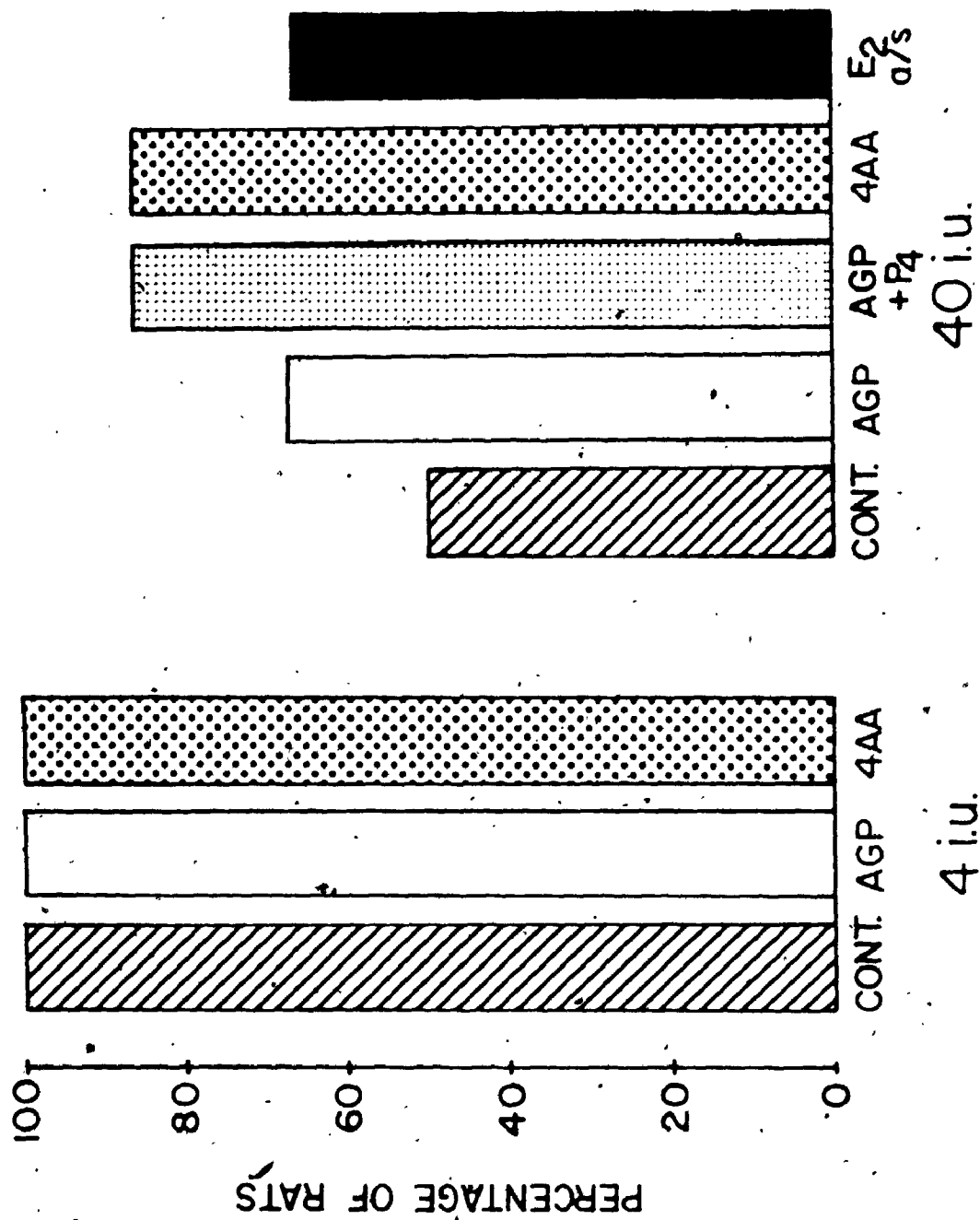
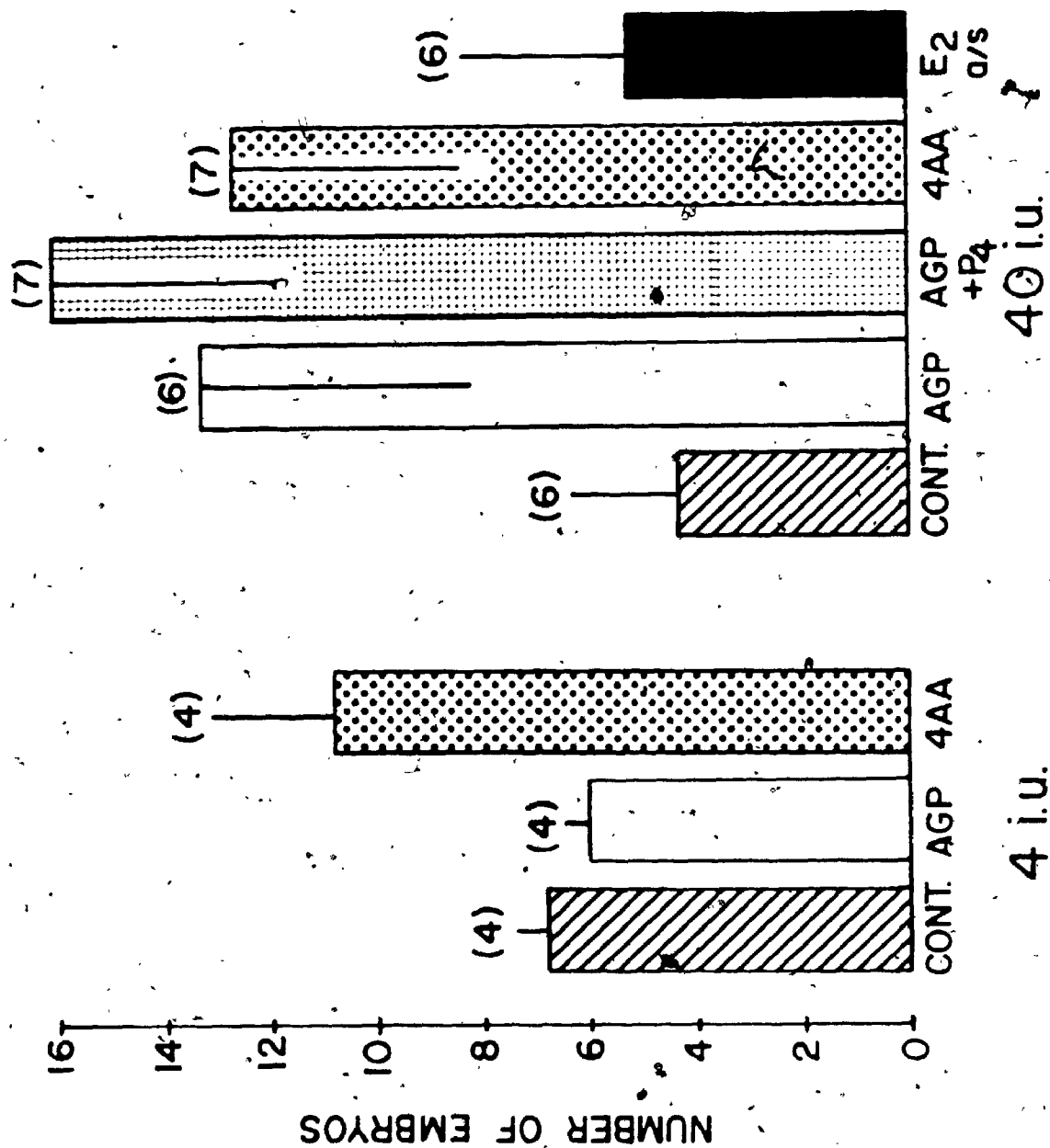


FIGURE 29

Mean embryo recovery rates on day 3 after anti-oestrogen treatment.  
(For details of treatments see Table 16). Numbers in brackets are  
the number of rats in each group.



embryo recovery rate for each treatment; almost all the embryos were recovered from the oviducts. Despite the apparent increase in embryo recovery rate following treatment with 4AA, AGP or AGP + P<sub>4</sub> the differences were not statistically significant due to the large variations between animals. The recovery rate of oocytes and their normality in 4 i.u. rats are given in Table 17. The percentage of abnormal and degenerate embryos was increased after treatment with 4AA ( $P < 0.01$ ). Injection of AGP had no effect on embryo normality.

Neither of the inhibitors nor the oestradiol a/s had any effect on ovarian weight (Figure 30). Figure 31 shows ovarian oestradiol content following the various treatments. After injection of AGP ovarian oestradiol content was significantly lower ( $P < 0.01$ ) in both 4 i.u. + AGP and 40 i.u. + AGP rats than in the respective controls. Levels in 40 i.u. + AGP and 40 i.u. + AGP + P<sub>4</sub> animals were reduced to values seen in control 4 i.u. rats. After implantation of 4AA values were not significantly different from those seen in control animals. Serum from 40 i.u. + E<sub>2</sub> a/s-treated rats bound 68% of a tritiated oestradiol tracer compared with 2% binding by the serum of control rats; this indicates that excess a/s was present. Ovarian oestradiol content could not be measured in the 40 i.u. + E<sub>2</sub> a/s rats due to the presence of excess circulating oestradiol a/s which interfered with the assay. The changes in oestradiol content are reflected in uterine weights (Figure 32). There was no significant difference between the 4 i.u. groups. Weights in the 40 i.u. + AGP and 40 i.u. + AGP + P<sub>4</sub> rats were significantly lower than the other 40 i.u. groups ( $P < 0.01$ ) whereas there was no significant difference between weights in the 40 i.u. control, 40 i.u. + 4AA and 40 i.u. + E<sub>2</sub> a/s rats.

TABLE 17

Normality of Oocytes Recovered on Day 3 of Pregnancy  
after Induction of Ovulation with 4 i.u. PMSG  
and Treatment with 4AA or AGP from Day 1 Onwards  
(n = 6 rats per treatment)

<u>Treatment</u>	<u>Embryos</u>	
	<u>Recovered</u>	<u>Degenerate</u>
Control	$6.8 \pm 1.3^a$	0.0 <sup>a</sup>
4AA	$5.0 \pm 1.3^a$	$2.7 \pm 1.1^b$
AGP	$6.8 \pm 0.7^a$	$0.8 \pm 0.4^a$

<sup>a,b</sup> within each column, means with the same superscript are not significantly different.

FIGURE 30

Ovarian weights on day 3 after anti-oestrogen treatment.  
(For details of treatments see Table 16).

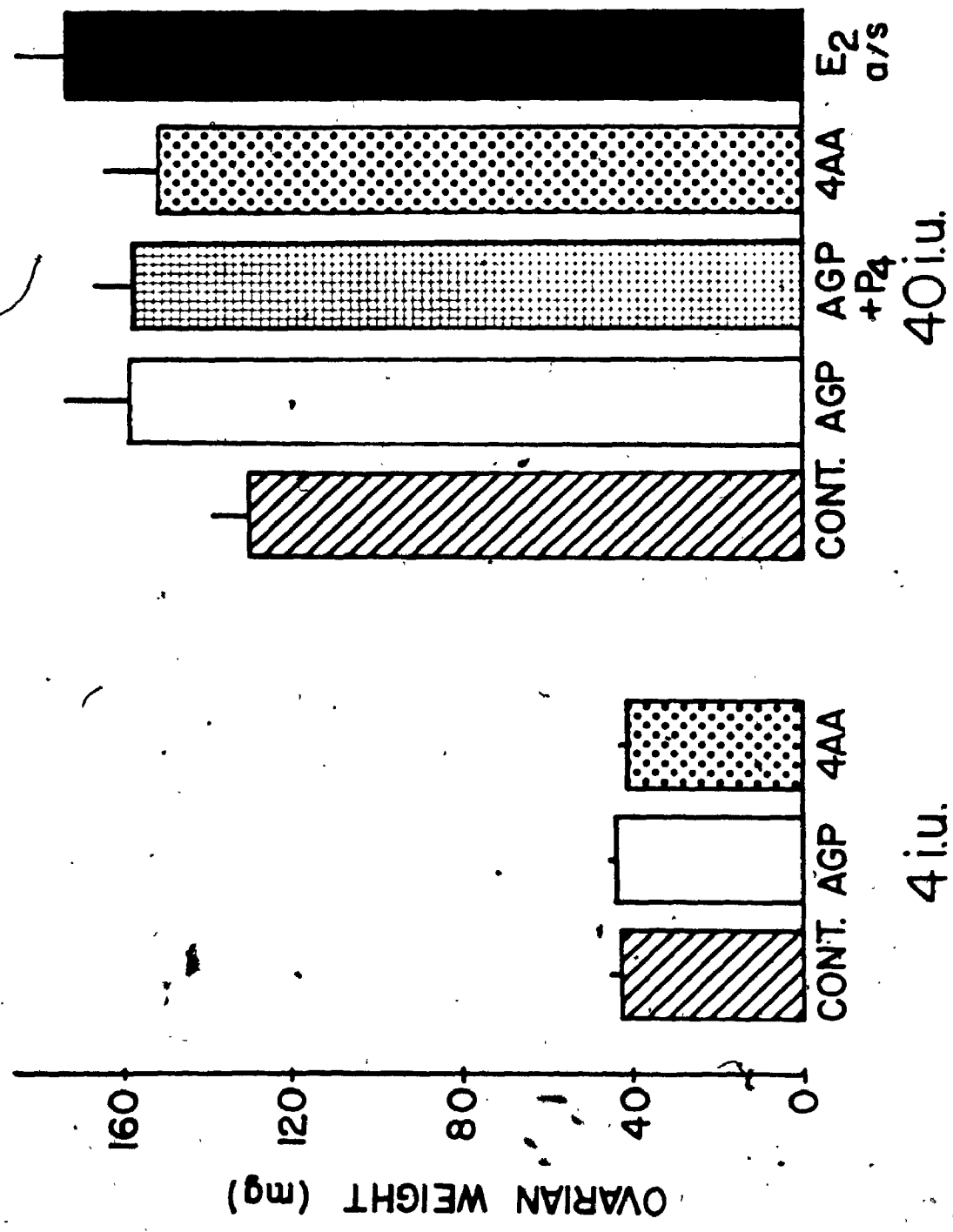


FIGURE 31

Ovarian oestradiol content and concentrations on day 3 after anti-oestrogen treatment. (For details of treatments see Table 16).



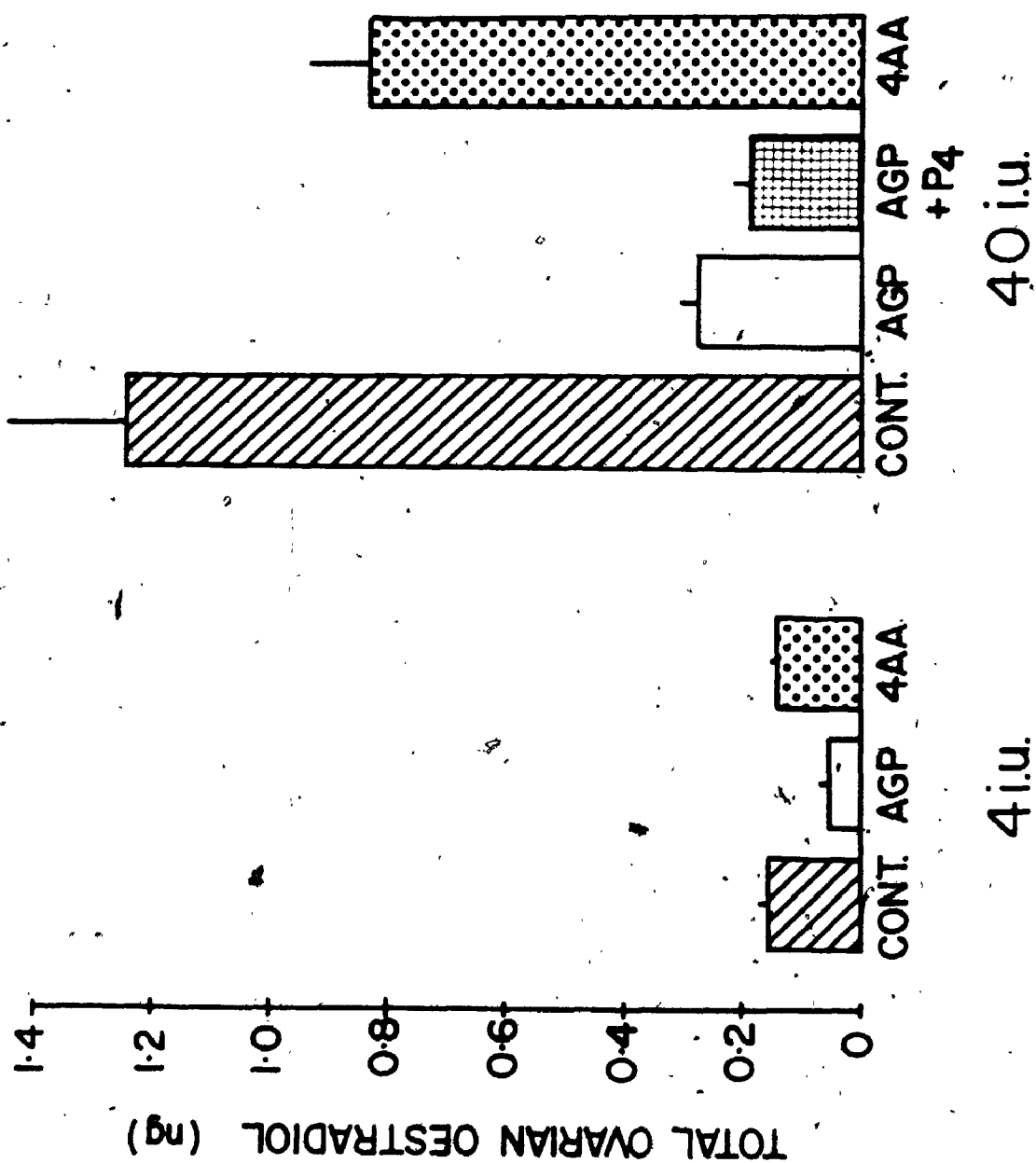
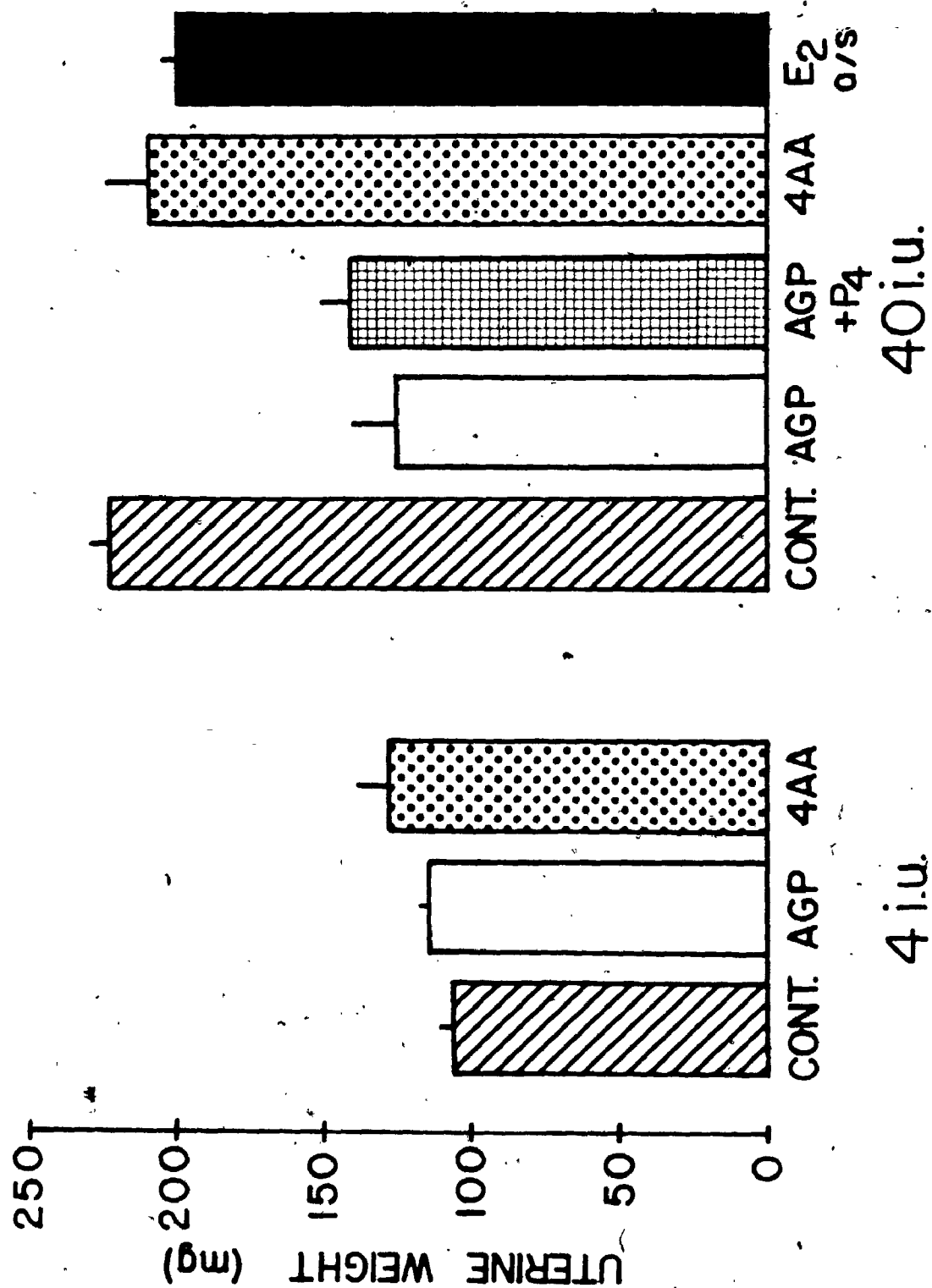


FIGURE 32

Uterine weights on day 3 after anti-oestrogen treatment.  
(For details of treatments see Table 16).



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Ovarian progesterone content is shown in Figure 33. With the exception of values in 4 i.u. + AGP rats which were significantly higher than 4 i.u. control rats treatment with inhibitor did not affect the progesterone content.

### 7.7. Discussion

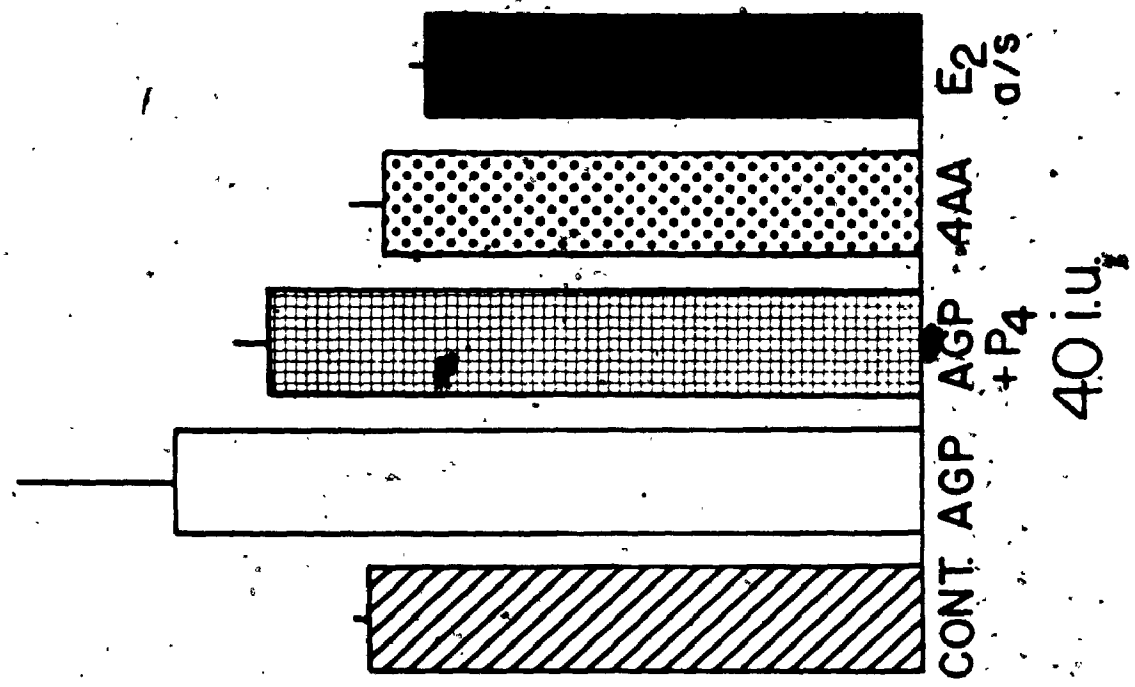
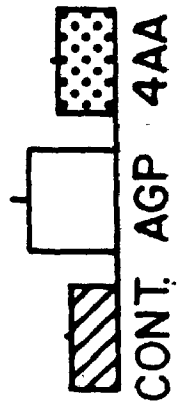
The experiments presented in this chapter suggest that several factors may contribute to the early loss of embryos seen after superovulation in immature rats. Based on observations in one-cell oocytes fertilization failure, both complete and partial, is higher in superovulated immature rats than in control animals. An earlier study utilizing a different superovulatory treatment regimen (Austin, 1950) had shown that 51% of all superovulated oocytes were unfertilized, but in that particular study no distinction was made between unfertilized degenerate and unfertilized one-cell oocytes. It was suggested that sperm transport might be a problem and that even when sperm were present in the oviduct the numbers were lower than in adult animals. However, since no control immature rats in which ovulation of physiological numbers of oocytes was induced were included, the possibility cannot be excluded that a lower number of sperm in the oviducts is normal in immature animals. In the present experiment total absence of sperm in the ampulla may have been the cause of fertilization failure in those rats which had no evidence of

FIGURE 33

Ovarian progesterone content and concentrations on day 3 after anti-oestrogen treatment. (For details of treatments see Table 16).

TOTAL OVARIAN PROGESTERONE (ng)

2500  
2000  
1500  
1000  
500  
0



19  
fertilization, but even in rats in which several oocytes were fertilized the proportion fertilized was still significantly lower than in control animals. This reduction might be attributed to a decreased number of sperm in the ampulla, genetic abnormalities in the oocyte, asynchronies between maturation, ovulation and mating or a less favourable endocrine environment, either within the follicle or in the oviduct immediately after ovulation.

Several authors have reported premature ovulation following injection of PMSG into rats and mice, but the exact timing of this premature ovulation has not been well established. Following injection of immature rats with 30 i.u. PMSG and 20 i.u. hCG 56 h later (Zarrow and Gallo, 1969) or a single injection of 30 i.u. (De la Lastra *et al.*, 1972) or 40 i.u. PMSG (Miller and Armstrong, 1981b), two distinct populations of oocytes have been observed on the third day after PMSG. Indirect evidence has suggested that the first ovulation 38-48 h after PMSG is a fairly discrete event, since oocytes recovered on day 0 are homogeneous, apparently normal oocytes always surrounded by a cumulus mass (Miller and Armstrong, 1981b). Most authors have therefore assumed that there are two discrete sets of ovulations on the second and third nights after PMSG (Fowler and Edwards, 1957; De la Lastra *et al.*, 1972; Park and Zarrow, 1972; De la Lastra and Forcelledo, 1973). Austin (1950) observed degenerate eggs in the oviduct within 24 h of the expected time of ovulation, but he did not consider the possibility that these may be from an early ovulation; rather, he considered that they represented abnormalities in the oocyte.

Kostyk et al. (1978) showed that at doses of 10-30 i.u. PMSG a certain percentage of immature rats ovulated within 24 h of the hormone and the percentage of rats ovulating did not significantly increase again until 48-72 h post PMSG. In contrast, doses of 60 i.u. PMSG resulted in a considerable increase between 24 and 48 h with only a slight increase after that time, although the mean number of oocytes increased with the time after PMSG. It was suggested that the exogenous gonadotrophin induced the release of oocytes from large follicles which happened to be present at the time of injection. Further evidence in support of this comes from the work of Sugawara et al. (1969) who showed that 40% of immature rats ovulated within 24 h of injection of hCG. A similar situation occurs in mice where superovulatory doses of PMSG cause ovulation within 20 h of injection, presumably as a result of the LH-like activity of PMSG (Stern and Schuetz, 1970; Fowler and Edwards, 1957). The oocytes from this early ovulation have been shown to develop normally after in vitro fertilization (Kaufman and Whittingham, 1972). In these mice a second ovulation occurs on the third night after PMSG.

Kostyk et al. (1978) suggested that administration of high doses of PMSG may result in the secretion of endogenous progesterone, which may then stimulate an early release of LH resulting in the 48 h ovulatory response, similar to administration of exogenous progesterone advancing the time of ovulation (Ying and Meyer, 1969b; Zarrow and Gallo, 1969). A similar premature release of LH was suggested by Park and Zarrow (1969) to explain the premature ovulation observed when oestradiol was administered at the same time as PMSG.



Several authors (Kostyk et al., 1978; De la Lastra et al., 1972) have suggested that the mechanisms involved in inducing the different ovulations may differ depending on the dose of gonadotrophin and the time following its administration. From experiments with chlorpromazine or hypophysectomy, De la Lastra et al. (1972) concluded that the ovulation within the first 48 h after PMSG was independent of the pituitary, whereas the second ovulation occurring between 60 and 72 h after PMSG was dependent on the pituitary. In the experiments of De la Lastra et al. (1972) most rats treated with 30 i.u. PMSG had ovulated by 36 h and the number of oocytes increased thereafter to plateau at 48 h. A further significant increase occurred between 60 and 72 h after PMSG. Thus the pattern observed was similar to that seen in the present investigation (Figure 21).

Due to the large variations between animals, in the present study it was difficult to establish exactly what pattern of ovulation was occurring. It was, however, clear that a large number of ovulations occurred in the superovulated rat at a time corresponding to that seen in control animals (between 2400 h on day 0 and 0600 h on day 1). Since the rise in degenerate oocytes approximately parallels the rise in oocytes recovered with a lag of approximately 36 h it may be concluded that degeneration does not occur for at least 36 h after ovulation. From their studies McCormack and Benrri (1970) concluded that naked oocytes must be at least 20 h post ovulation and, since loss of cumulus precedes degeneration, the oocyte may remain without cumulus for a further 16 h prior to degeneration. Zarrow and Gallo (1969) have suggested that oocytes from the premature ovulation are

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frequently expelled from the reproductive tract prior to 72 h after PMSG.

The experiments reported here suggest that in immature rats the early ovulation in response to superovulatory doses of PMSG occurs over a considerable time span, covering both the first and second nights after PMSG (Figure 21); this is not evident at sacrifice on day 0, since gross degenerative changes in the oocyte are not visible at that time.

A large number of ovulations occur in superovulated rats at a time corresponding to that seen in control animals, which suggests that the lower percentage of oocytes displaying pronuclear formation, compared to sperm penetration, is unlikely to be due to a later fertilization resulting from later ovulation. However, it is possible that, within the experimental variability, a small proportion of this large number of ovulations in superovulated rats occurred relatively late and these oocytes had not yet progressed to the pronuclear stage by the time of sacrifice; pronuclear formation is complete within 10-12 h of insemination in vitro (Toyoda and Chang, 1974).

Since degeneration may not occur for at least 36 h after ovulation, the decreased fertilization rate seen in the superovulated rats may be partially due to oocytes ovulated prior to the night of day 0 to day 1 that are still one-cell and apparently normal, but too aged to undergo fertilization.

Conclusions regarding the effects of the follicular or oviductal endocrine environments on fertilization cannot be drawn

from the present set of experiments, but changes in the endocrine environment, particularly elevated levels of sex steroids, have been shown in immature superovulated rats (Chapter 6) and these may have effects on the fertilization process.

The actual timing of ovulation relative to the LH surge appears to be an important factor for normal fertilization to occur.

Niwa and Chang (1975), working on immature rat oocytes cultured in vitro showed that fertilization of 'early' oocytes (collected 0-4 h after hCG and prior to normal ovulation) or of 'late' oocytes (collected 12-14 h after ovulation) resulted in sperm penetration, but the sperm head failed to be transformed into a male pronucleus. Thus in the present experiment the lower percentage of superovulated oocytes showing pronuclear formation compared to sperm penetration could be explained by a failure of transformation of the sperm head to a pronucleus.

The fertilizability of the oocyte may outlast its fertility and its capacity to develop into a normal embryo (reviewed by Austin, 1970). This most frequently occurs when rats are mated or artificially inseminated after the time of ovulation. Under these circumstances the female pronucleus may fail to form or may fragment (Blandau, 1952). A similar result might be expected when oocytes are ovulated early relative to the time of mating, as may be the case in the present study. Sperm penetration would occur but pronuclear formation might subsequently fail.

Following transfer beneath the bursa of a synchronized recipient, the percentage of oocytes which developed to fetuses was

not significantly different when recovered from the oviducts of control or superovulated donors (Table 12). This strongly suggests that oocytes recovered from superovulated donors are normal and the failure to develop, seen in previous studies (Miller and Armstrong, 1981b), is not attributable to defects in the oocytes. Miller and Armstrong (1982) found that in superovulated rats ovariectomy within 36 h of the time of mating resulted in a large proportion of embryos implanting. They could not however determine if the embryos which did not implant were abnormal. In the present study oocytes were selected at random from the large number recovered immediately after ovulation and a decrease in the survival rate would be expected if some of the oocytes were abnormal.

It has been suggested that an early rise or prolonged elevation of preovulatory oestrogen levels may cause increased preimplantation death of embryos, through a direct effect on the preovulatory oocyte (Butcher and Pope, 1979). The oestradiol concentrations in both serum and ovaries are elevated in the superovulated rat prior to ovulation (Miller and Armstrong, 1981b; Chapter 6) and yet the present study shows no increased level of oocyte abnormalities relative to control. The earlier results of Butcher and Pope (1979) might therefore only be applicable in cases of prolonged exposure to high concentrations of oestrogen, due to delays in ovulation. Also in that study no direct assessment was made of the normality of the oocytes and no direct evidence of effects on the preovulatory oocyte was presented. In addition the effects of oestradiol on the oviductal environment and hence early embryonic development were not considered.

When ovarian oestradiol content was reduced to that seen in control animals by means of an inhibitor of oestradiol synthesis (AGP), embryo recovery rate was similar to that observed earlier in PMSG a/s rats. That embryo survival was not more effectively increased may be attributable to several factors. AGP is a pharmacological blocker of cytochrome P<sub>450</sub>-dependent enzymes and consequently inhibits several enzymes involved in steroid biosynthesis (Kahnt and Neher, 1966), including the initial step of cholesterol side chain cleavage (Cohen, 1968) and aromatase activity (Santen et al., 1978; Graves and Salhanick, 1979). The synthesis of many other steroids in addition to oestradiol is therefore inhibited and some of these steroids may be beneficial to embryo survival. Long-term daily injections of AGP, prior to mating, have been shown to depress fertility and decrease litter size (Eversole and Thompson, 1974). In the present study in control 4 i.u. rats, AGP had no effect on embryo normality, probably because of the shorter length of time over which it was injected; however another, more specific, inhibitor of aromatase activity, 4AA, caused an increase in abnormal embryos. The reason for this is unknown but it is possible that treatment with AGP may resemble the situation seen after ovariectomy, when embryo development can proceed to the blastocyst stage without administration of exogenous hormones (Alden, 1942b; Wu et al., 1971) or following injection of progesterone alone (Alloiteau and Psychoyos, 1966). It has, however, been reported that administration of progesterone to ovariectomized rats has a deleterious effect on the transformation of the morula to the blastocyst (Dickmann, 1970). AGP will prevent the

synthesis of most steroids, whereas 4AA, being a more specific inhibitor, may cause the accumulation of other steroids which may be detrimental to embryo normality. Alternatively 4AA may itself be embryotoxic.

Ovarian progesterone content (Figure 33) suggests that neither inhibitor was affecting synthesis of this steroid, whereas AGP was ~~more~~ effective than 4AA in reducing ovarian oestradiol content (Figure 31). Since AGP is widely used as an inhibitor of cholesterol side chain cleavage the lack of effect on progesterone concentrations was unexpected. However the blockade of early steroid biosynthesis may only be partial (Santen et al., 1979) and both plasma and ovarian progesterone concentrations start to recover 6 h after AGP injection (Glasser et al., 1972; Inaba and Wiest, 1980), although preinjection values are not reached for 48 and 96 h respectively.

Injection of excess of a specific oestradiol antiserum did not improve embryo recovery, which suggests that the detrimental effects of oestradiol may have occurred prior to day 1 when anti-oestradiol treatment was initiated. In addition the detrimental effects may involve compounds other than, or in addition to, oestradiol.

It has been shown that the detrimental effects of high levels of oestrogen include accelerated transport of embryos and their subsequent expulsion (Greenwald, 1961; Banik and Pincus, 1964; Ortiz et al., 1970). It was therefore of interest to try to retain the embryos within the uterus by means of ligatures. At sacrifice on day 4 the number of embryos recovered was increased (Table 15)

and a single ligature at the uterotubal junction was more effective than two ligatures, both in terms of the percentage of rats from which embryos were recovered and in the percentage of normal embryos. This may reflect less trauma and adhesions when only one ligature was tied. In both 4 and 40 i.u. rats the proportion of abnormal embryos was increased in the presence of a ligature (Figure 25). In addition to the possibility of increased trauma, the presence of a ligature may change the oviductal environment or it may prevent the expulsion of degenerate eggs, which are somehow recognized by the oviduct and subsequently removed. In particular the tying of two ligatures caused an increased fluid accumulation within the oviduct. In the ampulla the fluid has been shown to be secreted by the oviductal wall (Shalgi et al., 1977); thus the presence of ligatures will result in a build-up of fluid which may itself be detrimental. Any changes in the composition of the fluid as a result of the ligatures is unknown, but the possibility exists that harmful metabolites may accumulate.

No count of the number of ovulations was made in this experiment, but the number of embryos recovered from the double-ligated oviduct of 40 i.u. rats was considerably lower than would be expected from the number of ovulations observed on day 1 (Chapter 6). The presence of a double ligature therefore caused the disappearance of a greater proportion of embryos from superovulated rats; the reason is unknown, but the tying of a double ligature did decrease the percentage of normal embryos recovered (Figure 27), so the possibility exists that in these oviducts a greater proportion of embryos are

totally degenerating, or degenerating to an unrecognizable state.

In none of the present studies was an assessment of the chromosomal normality of the oocytes made, but following transfer fetuses developed equally well from control or superovulated oocytes, suggesting that there are no detrimental chromosomal abnormalities in the superovulated oocytes. Similarly, in mice Gates, (1965) has suggested that the oocytes produced from superovulated donors are genetically normal, as assessed by in vitro development from the 2-cell to blastocyst stage; these blastocysts gave rise to viable fetuses when transferred to suitable recipients.

The increased number of fetuses recovered after transfer of oocytes collected from the follicles of control rats compared to oviductal oocytes (Table 12) may reflect the better timing of transfer in these animals. Fertilization begins within 2 h after the median time of ovulation or probably immediately following ovulation in some individual animals (Shalgi and Kraicer, 1978). Immature female rats injected with 4 i.u. PMSG ovulate between 0030 h and 0530 h (Figure 21). Since transfers were performed between 0300 h and 0600 h some oocytes could, in theory, have been up to 5 h post ovulation at the time of transfer. In contrast, follicular oocytes were collected from control animals which had not yet ovulated or had only partially ovulated and in this case ovulation was presumed to be imminent. The timing of collection and fertilization in the recipient was therefore probably closer to the normal situation of ovulation and fertilization in these animals. A lower percentage of oocytes collected within 5 h after ovulation developed compared to follicular



oocytes collected at the first polar body stage (Noyes, 1952); this was interpreted as a rapid deterioration of oocytes after ovulation. This explanation is not supported by the work of Niwa and Chang (1975), who showed that eggs recovered 4-14 h after ovulation could be penetrated by spermatozoa in vitro and develop into apparently normal 2-cell eggs, although further development was not assessed. Therefore a delay of 5 h (the maximum that could be encountered in the present study) would probably not be detrimental.

In contrast to control rats, in the superovulated animals the percentage of follicular oocytes which survived to fetuses was not significantly different from that of oviductal oocytes and it was significantly lower than the survival rate of the control follicular oocytes (Table 12). The reason for this difference is unknown, although it seems probable that the follicular oocytes transferred from the superovulated donors represent a more heterogeneous group than those from the control animals. Almost all superovulated donors had oocytes surrounded by cumulus in the oviducts, but the numbers varied. Follicular oocytes collected from superovulated donors could theoretically belong to at least two classes of oocytes: those from follicles about to ovulate and those from follicles which would not ovulate but luteinize (Wilson and Zarrow, 1962; Miller and Armstrong, 1981b). In the first class high success rates might be expected, but the normality of oocytes in the second class has not been assessed. In the present experiment it was not possible to assess the contribution of each class to the pool of follicular oocytes; large follicles were punctured at random to collect oocytes.

The normality of oocytes collected earlier than ~ 67 h after PMSG in immature rats has been questioned. Schuetz (1971) has suggested that oocyte 'maturation' and follicular development are made asynchronous by precocious ovulation (24 h early) and this interferes with the course of normal pregnancy. Kaufmann and Whittingham (1972) have shown, using in vitro fertilization techniques and transfer to a synchronized recipient, that oocytes ovulated by mice within 14 h of an injection of PMSG are as viable as those recovered at the normal time. Miller and Armstrong (1982) observed that a low proportion of rats superovulated with 40 i.u. PMSG would mate 24 h earlier than normal (i.e., 40-46 h post PMSG) and implantation was not observed in any such animals which remained intact. However, if such rats were ovariectomized about 12 h after mating then 50% became pregnant. This suggests that oocytes from these rats were normal and the pregnancy failure in the intact animal was probably due to a failure of the ovary to provide a suitable endocrine environment to permit implantation. In the present study the numbers of oocytes transferred after collection at 26.5-29.5 h or 42.5-45.5 h after PMSG were relatively low, but nevertheless it appears that oocytes collected at 26.5-29.5 h are as capable of developing into foetuses as are those collected 66.5-69.5 h after PMSG. The timing of early ovulations in the immature superovulated rat appears to be very variable (Figure 21) and oocytes collected at 42.5-45.5 h may not all be freshly ovulated even though they remain in cumulus, whereas at 26.5-29.5 h all oocytes are collected relatively close to their time of ovulation.

Cumulus cells undoubtedly assist in the pick-up of ovulated oocytes by the fimbriae of the infundibulum, but oocytes without surrounding cumulus cells (denuded with hyaluronidase) can also be picked up and develop into foetuses, although less efficiently (43.8% cumulus enclosed oocytes vs. 14.3% denuded oocytes). In addition to a probable reduction in efficiency of pick-up, when oocytes are denuded with hyaluronidase, it has been shown that, at least in the mouse (Miyamoto and Chang, 1972), in vitro fertilization rates are decreased significantly. A similar situation in the rat could result in a decreased fertilization rate and subsequently, a decreased proportion of denuded oocytes developing to foetuses in the present study. In the present study the fact that no oocytes without cumulus developed probably indicates that those oocytes had been ovulated for some hours and were too old to undergo fertilization, even though degenerative changes and fragmentation had not yet occurred. Oocytes without cumulus cells attached may be at least 20 h post ovulation (McCormack and Bennin, 1970).

The studies presented in this chapter show that preimplantation embryo losses may result from a less exact timing of ovulation causing asynchrony of ovulation and mating, decreased fertilization rate and a hostile oviductal environment. The relative contribution of each has not been assessed but oestrogen has been implicated in the formation of the unsuitable oviductal environment. The oocyte itself appears to be normal.

## CHAPTER 8

### IMPLANTATION FOLLOWING SUPEROVULATION

#### 8.1 Introduction

Under physiological conditions, excluding lactation, the presence of a mature blastocyst in the uterus coincides with a period of uterine receptivity (Psychoyos, 1973a), at which time implantation is initiated. There are therefore two requirements for successful implantation - a mature blastocyst and a receptive uterus. An abnormality in either one will cause pregnancy failure. Compared to control animals, concentrations of circulating oestradiol in SOV a/s rats are elevated, particularly around the time of ovulation and during the first days of pregnancy (Chapter 6). After ovulation progesterone concentrations rise significantly higher in the SOV a/s rats (Chapter 6). Embryo development in vivo and in vitro has been shown to be detrimentally affected by oestrogen-dominated oviducts (Cline et al., 1977; Stone et al., 1977) and high concentrations of oestradiol have been shown to inhibit the development of uterine sensitivity (Yochim and De Feo, 1963). In addition, Butcher et al. (1969a) have suggested that high preovulatory levels of oestradiol may independently affect both the oocyte and the uterus. All these studies suggest that the altered endocrine environment in SOV a/s rats may be detrimental to embryo development both prior to and at the

time of implantation.

In Chapter 6 it was shown that blastocysts could be recovered from 100% of SOV a/s rats on day 5 of pregnancy, but only 50% of these animals had implantation sites on day 8. The present set of experiments was designed to investigate whether the failure to implant in 50% of SOV a/s rats was due mainly to abnormalities in the blastocysts, or whether it was the result of an unsuitable uterine environment which prevented implantation and subsequent pregnancy.

## 8.2 Blastocyst Normality

### 8.2.1 Methods

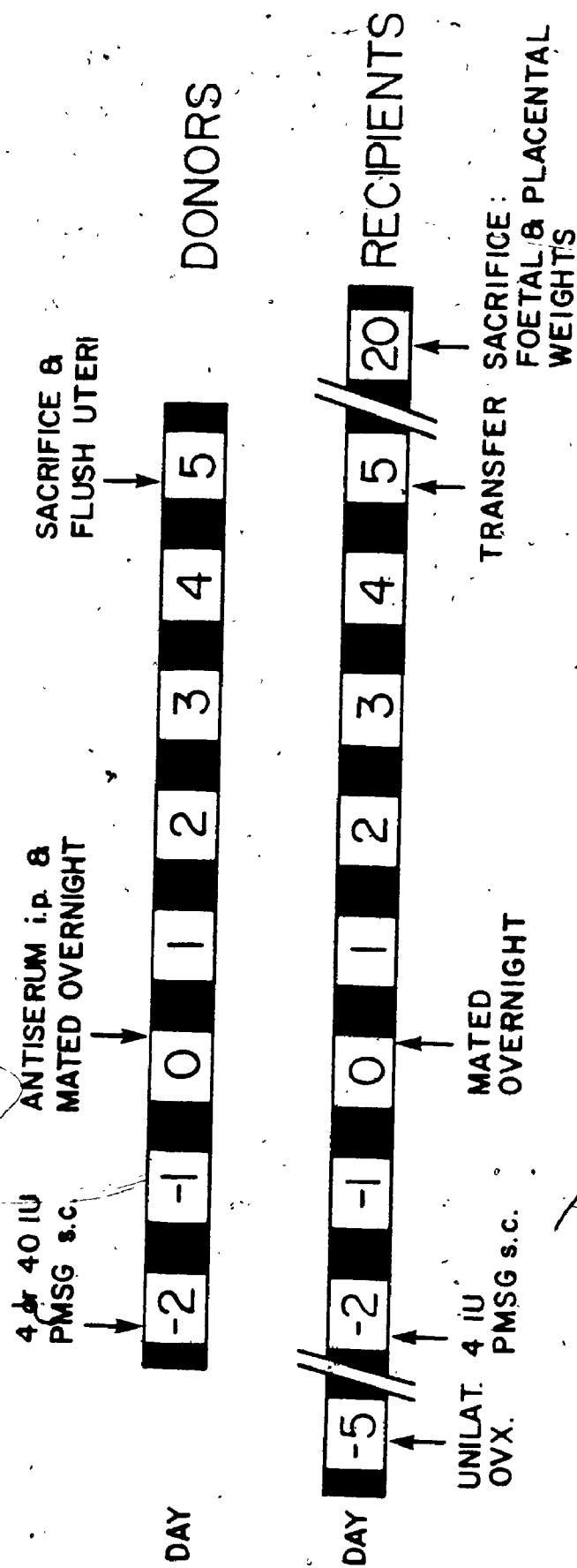
Immature rats to be used as recipients were unilaterally ovariectomized by a flank incision at body weight 50-60 g. Figure 34 shows a schematic representation of the procedure used for the transfer experiments. Recipient rats and 50% of donor rats (control) received 4 i.u. PMSG s.c. at 0830 h on day -2. The remaining superovulated donors were injected with 40 i.u. PMSG at the same time and 0.2 ml PMSG a/s at 1800 h on day 0 (SOV a/s group). All donors and recipients were mated.

On day 5 between 0900 h and 1300 h donor rats were sacrificed by cervical dislocation and the uterine horns were flushed as described previously (Chapter 6), using sterile DBS + 5% rat serum (heat-inactivated, charcoal-treated). Blastocysts recovered were

FIGURE 34

Schematic representation of the procedure for uterine transfers in immature rats.

Light and dark areas indicate periods of light and darkness respectively. The numbers refer to the day of the experiment (where day 1 = first day of pregnancy).



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counted and any grossly abnormal blastocysts discarded. Control and SOV a/s donors were killed alternately and multiple transfers were carried out from a single donor when the number of blastocysts was sufficient. Recipient rats were anaesthetized with tribromo-ethanol solution (2%, 0.04 ml/g body weight) and the previous incision was reopened. The uterus was exposed, care being taken to avoid handling, and using a 25-gauge needle a small incision was made in the uterus, close to the uterotubular junction. Four to seven blastocysts were transferred by means of a heat-polished glass pipette attached to a mouth tube.

Recipient rats were then sacrificed on day 20 and the total number of foetuses, together with the number of live foetuses, was recorded in both the control (nontransferred) and transfer horns. Total foetal and placental weights were also recorded for each horn. Any rats not pregnant in the control horn were excluded from the results.

Recipients were classified into two groups according to the donor of the blastocysts which were transferred. Comparisons were made within the groups between the control and transfer horns or between the transfer horn of the two groups. The proportion of blastocysts recovered following transfer was compared using a  $\chi^2$  test; comparisons of foetal and placental weights were made using a t-test.

To determine if success rates were influenced by the size of the recipient, a small number of blastocysts from 4 i.u. donors were transferred to adult unilaterally ovariectomized mated rats, using the same techniques described for immature recipients.



### 8.2.2 Results

Results are from 32 recipients receiving blastocysts from 4 i.u. (4 i.u. blastocysts) and 36 recipients receiving blastocysts from SOV a/s donors (SOV a/s blastocysts). In the transfer horn 71.9% of recipients receiving 4 i.u. blastocysts were pregnant, which was not significantly different from the 69.4% observed in recipients receiving SOV a/s blastocysts. On day 20 44.3% of transferred 4 i.u. blastocysts were recovered compared to 41.9% of transferred SOV a/s blastocysts (81/183 vs. 88/210), whereas the percentages of viable fetuses were 36.1% and 31.9% from 4 i.u. and SOV a/s blastocysts, respectively (66/183 vs. 67/210). Neither of these differences was statistically significant.

Table 18 shows the number of fetuses and foetal and placental weights for both groups of rats. Comparing the transfer horn with the control horn in each group the mean foetal weights were not significantly different, whereas the mean placental weight in the transfer horn of rats receiving blastocysts from SOV a/s donors was significantly ( $P < 0.01$ ) higher than the control. This difference was not significant for recipients receiving 4 i.u. blastocysts.

On day 20 5/6 adult recipients were pregnant and 15 live fetuses were recovered from 22 transferred blastocysts (68.2% success rate). This is significantly higher ( $P < 0.01$ ) than the 36.1% success rate seen in immature recipients.

TABLE 18

Blastocysts Recovered as Foetuses and Placental Weights (mean  $\pm$  S.E.M.)  
 following Transfer of Blastocysts, from 4 i.u. or SOV a/s Donors,  
 to the Uteri of Synchronized Immature Recipients

	4 i.u. Donors		SOV a/s Donors	
	Control Horn	Transfer Horn	Control Horn	Transfer Horn
Mean number of embryos transferred	-	5.7 $\pm$ 0.2	-	6.0 $\pm$ 0.3
Mean number of foetuses recovered	8.7 $\pm$ 0.4	2.5 $\pm$ 0.4	8.3 $\pm$ 0.3	2.5 $\pm$ 0.3
Mean number of live foetuses recovered	8.0 $\pm$ 0.4	2.1 $\pm$ 0.3	7.7 $\pm$ 0.3	1.9 $\pm$ 0.3
Mean foetal weight (g)	1.7 $\pm$ 0.1	1.5 $\pm$ 0.1	1.8 $\pm$ 0.1	1.8 $\pm$ 0.1
Mean placental weight (mg)	419 $\pm$ 14	423 $\pm$ 16	449 $\pm$ 11	536 $\pm$ 28

### 8.3 Uterine Normality

#### 8.3.1 Methods

Immature rats were injected with 4 or 40 i.u. PMSG on day -2 and the superovulated rats received 0.2 ml a/s at 1800 h on day 0. All rats were then mated. On day 5 between 1330 and 1530 h animals were anaesthetized with ether and a uterine horn was exposed via a flank incision. To traumatize the endometrium a 25-gauge needle with a burred tip was introduced into the uterine lumen through an incision close to the uterotubal junction. The needle was inserted towards the cervical end of the uterus and then withdrawn while scratching the antimesometrial surface (Yochim and De Feo, 1962).

On day 10 animals were sacrificed by cervical dislocation and the uterine horns were dissected free of fat. The horn on the traumatized side was weighed and on the control side the number of implantation sites, when present, together with the total implantation site weight were recorded. A traumatized uterine horn weight greater than 200 mg was considered to be evidence of decidualization (De Feo, 1963).

Comparisons between the proportions of rats pregnant, with and without decidualization, were made using a  $\chi^2$  test; deciduomata and implantation site weights were compared by a t-test.

#### 8.3.2 Results

The occurrence of pregnancy and decidualization in 4 i.u. vs. SOV a/s-treated rats is shown in Table 19. There were significant

TABLE 19

The Occurrence of Pregnancy and Decidualization  
in 4 i.u. or SOV a/s Rats (for details of treatment see text;  
figures in brackets represent percentage of total rats)

	<u>Number of Rats Treated with</u>	
	<u>4 i.u.</u>	<u>SOV a/s</u>
Total	29	51
Pregnant	24 (82.8)	19 (37.2)
Deciduomata	29 (100.0)	24 (47.1)
Pregnant with deciduomata	24 (82.8)	13 (25.5)
Not pregnant with deciduomata	5 (17.2)	11 (21.6)
Pregnant with no deciduomata	0	6 (11.8)
Not pregnant with no deciduomata	0	21 (43.1)

differences between the two treatments, both in the percentage of rats pregnant ( $P < 0.005$ ) and in the percentage of rats showing decidualization ( $P < 0.005$ ). Comparing only pregnant rats, the percentage of 4 i.u. rats showing decidualization was still significantly higher ( $P < 0.025$ ). Similarly, comparing only nonpregnant rats, there was also a significant difference ( $P < 0.05$ ) in the percentage with deciduomata (5/5 vs. 11/32). Comparing all rats with evidence of decidualization (traumatized horn weight  $> 200$  mg), the mean deciduomata weight ( $\pm$  SEM) of control rats was  $897 \pm 39$  mg, which was significantly higher ( $P < 0.005$ ) than the value of  $667 \pm 65$  mg observed in SOV a/s rats.

The mean implantation site weight ( $\pm$  SEM) for control rats was  $63.8 \pm 1.6$  mg compared to  $43.5 \pm 5.0$  mg for SOV a/s-treated rats showing decidualization and  $34.3 \pm 4.3$  mg for SOV a/s-treated rats with no decidualization. The difference between control and both SOV a/s groups was significant ( $P < 0.001$ ), whereas the difference between the two SOV a/s groups was not significant.

#### 8.4 Discussion

The results presented in this chapter show that blastocysts from 4 i.u. or SOV a/s-treated donors are equally capable of developing into fetuses when transferred to a 'physiologically normal' recipient. In an earlier study 100% of rats receiving 4 i.u. PMSG and only 50% of those receiving 40 i.u. PMSG + a/s had

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evidence of implantation on day 8, whereas all animals had blastocysts in the uterus on day 5 (Chapter 6). This failure to implant could have been due to latent abnormalities in the blastocyst, asynchrony of the uterus and blastocyst or to a failure of the uterus to provide a suitable environment for implantation.

Since foetuses which develop from transferred blastocysts were not significantly different in weight from the foetuses in the control horn (Table 18), it is concluded that transfer does not detrimentally affect implantation or foetal development. The higher mean placental weight in the transferred horn of recipients receiving blastocysts from SOV a/s donors was due to the presence of several rats with only a single pregnancy and a single enlarged placenta in this horn. Surgical reduction of the litter size during the first half of pregnancy has been shown to enhance significantly the growth of the remaining placentae and foetuses (Crosskerry and Dobbing, 1978) and placental weight is known to vary inversely with litter size (Csapo and Wiest, 1973). However, the transfer horn of recipients receiving blastocysts from 4 i.u. donors also had a single pregnancy on several occasions but the placentae in these animals were not enlarged, and there is no obvious explanation for this difference.

The percentage of transferred blastocysts which developed into foetuses was lower than the value of approximately 60% reported by previous workers working with adult pseudopregnant rats (Dickmann and Noyes, 1960; Mantalenakis and Danezis, 1968). In the same studies the success rate was approximately 70% if the day 5 blastocysts were transferred to day 4 uteri. It may therefore be that the timing

of transfer in the present experiments was not optimal and higher success rates might have been achieved if the transfers had been made to day 4 uteri.

The uterus of the immature rat induced to ovulate with PMSG may also be more sensitive to handling and trauma than that of the adult cycling rat. In addition, the shorter length of the uterus in the immature animal makes it inevitable that a larger percentage of the uterus will be traumatized by the procedure involved in introducing the blastocysts. Evidence that the lower recovery rate may indeed be due to the use of immature rats as recipients comes from the small study in which recipients were unilaterally ovariectomized adult rats; in these animals 68% of transferred blastocysts were recovered.

Despite the lower success rate, this study does provide evidence that those embryos which develop to the blastocyst stage and remain in the uterus on day 5 in SOV a/s-treated rats are normal, suggesting that asynchrony or an unsuitable uterine environment may be responsible for the failure to implant in a large proportion of SOV a/s-treated rats.

The decidualization studies suggest that, since there is a significant difference in both the percentage of rats pregnant and the percentage showing decidualization (Table 19), the larger number of SOV a/s rats not pregnant might be related to a reduced ability of the uteri of these animals to undergo decidualization, at a time when the eggs have reached a stage of development appropriate for implantation. Whether or not the uterus is sensitive at some other time cannot be determined from this study, but De Feo (1963) has shown that

in adult pregnant or pseudopregnant rats a scratch trauma is capable of inducing decidualization over a period of approximately 45 h (1200 h day 4 to 0900 h day 6). Thus in the present study it is likely that if asynchrony exists it must be greater than 24 h. Alternatively, the hormonal environment to which the uterus was exposed may have rendered the uterus completely refractory to a deciduogenic stimulus in a large number of SOV a/s rats. Earlier work by Armstrong and Greep (1965) showed that the uterus may be completely refractory, since they were unable to produce deciduomata in any rats by scratching on days 3-7 after hCG, following priming with 50 i.u. PMSG 2½ days earlier. These authors showed that the failure to form deciduomata was probably not due to a deficiency of prolactin or LH, but rather to excess secretion of some inhibitor of uterine sensitivity to trauma. The possibility that this inhibitor was oestrogen was considered.

Zarrow et al. (1968) observed deciduomata in 100% of rats injected with 45 i.u. PMSG and traumatized on day 6 after the hormone, whereas Koch and Oettel (1977) found decidualization to be inhibited in rats treated with 32 i.u. PMSG. The present study is therefore intermediate between these two with decidualization being inhibited in ~ 50% of animals.

A significantly lower percentage of nonpregnant SOV a/s rats (compared to controls) showed decidualization, which suggests that whereas pregnancy failure in controls might be attributable to embryo failure (all control rats showed decidualization), an unsuitable uterine environment might be the cause of pregnancy failure in a proportion of SOV a/s rats.



As discussed in Chapter 6 both progesterone and oestradiol are essential for implantation in the rat, and ovarian hormone secretion during the oestrous cycle and early pregnancy normally ensures that uterine receptivity coincides with the presence of a mature blastocyst (Psychoyos, 1973b). Yoshinaga and Greep (1970) have shown that in pseudopregnant rats precocious sensitivity of the uterus can be produced by increasing progesterone levels for 3 days prior to oestrogen treatment on the day of trauma. In contrast, oestrogen treatment prior to trauma reduced the uterine sensitivity. Similarly, Sartor *et al.* (1978) have shown that a single high dose of oestradiol (10 µg) on day 4 of pregnancy leads to abnormalities during implantation. These authors showed that the oestradiol accelerated the processes leading to the uterine receptive phase and decreased the level of polyploidy reached in the antimesometrial cells. Both these studies confirm the suggestion of Courrier (1950) that the optimal conditions for implantation are produced by a delicately balanced synergism between the actions of oestrogen and progesterone.

In SOV a/s rats serum concentrations of oestradiol were higher than control animals over the period days 1-5, whereas ovarian content was not different (Figures 8 and 9). Progesterone concentrations in both ovaries and serum from SOV a/s rats were also higher. Thus the balance between these two hormones may well have been disturbed.

In SOV a/s rats a small number of rats (6/51) had implantation sites in the absence of decidualization in the traumatized horn, suggesting that, at least in this group, implantation can occur in a uterus which is unable to undergo decidualization in

response to an artificial decidualogenic stimulation at the time implantation was expected to occur. It is possible therefore that in the SOV a/s group, there is a complete spectrum of animals ranging from those in which implantation occurs at the normal time and which show decidualization in response to trauma, through those with a small delay in implantation and reduced decidualization to animals with an even greater delay which showed no decidualization. The weight of the implantation site would decrease with increasing delay, although apparently not to a significant degree. The mean implantation site and deciduomata weights of SOV a/s-treated animals were significantly lower than those of control rats, suggesting that a delay may have occurred in all SOV a/s rats. However, as discussed earlier, for implantation to occur in the absence of a decidualization a delay of at least 24 h would be required.

The results of the decidualization study therefore confirm the results of the transfer study, in which blastocysts from SOV a/s rats were shown to be able to develop normally, suggesting that the pregnancy loss was due to an unsuitable uterine environment or asynchrony between the embryo and the uterus. The decidualization study indicates that in a large proportion of SOV a/s rats the uterus is unable to undergo decidualization at the normal time, which may account for the pregnancy failure in these rats. Superovulation may therefore cause changes in the uterine environment which cannot be totally reversed by PMSG antiserum.

## CHAPTER 9

### SUMMARY AND CONCLUSIONS

Using the immature rat as a model for the infertility that results from superovulation several aspects of postovulatory development were studied. The results presented in this thesis, suggest that superovulation with exogenous gonadotrophins can produce a large number of oocytes which are capable of embryonic and foetal development if such development occurs in a 'physiological normal' environment.

Although oocytes collected from superovulated rats immediately after ovulation were normal, as assessed by transfer to synchronized recipients (Table 12), pregnancy failed early in rats superovulated with 40 i.u. PMSG. The first defect observed in the PMSG superovulated rat was a significant decrease in the proportion of 1-cell oocytes fertilized (Table 14). This may reflect the long time span over which ovulation was observed in the superovulated rat in both this study (Figure 21) and previous studies (De la Lastra *et al.*, 1972; Kostyk *et al.*, 1978). In addition, an earlier study had suggested that sperm transport may be affected by superovulation (Austin, 1950).

Superovulation with PMSG caused elevations in both pre- and postovulatory oestradiol concentrations in the serum and ovaries (Figures 8 and 9), and these elevations appeared to be detrimental to

later embryonic development.

Elevation of postovulatory oestrogen caused a rapid loss of embryos, similar to that seen in previous studies, where injection of oestrogen on day 1 of pregnancy resulted in accelerated transport of oocytes (Greenwald, 1961; Banik and Pincus, 1964; Ortiz et al., 1979). Ligation of the uterotubal junction, to prevent accelerated transport of embryos into the uterus, did increase the number of embryos recovered from the oviduct on day 4 (Table 14), but the proportion of abnormal embryos was also increased (Figure 25). A similar increase in the proportion of abnormal embryos was observed in control rats with an oviductal ligature, which suggests that the presence of the ligature was itself detrimental to embryo normality.

2. The use of a PMSG antiserum partially overcame the early embryo loss in PMSG superovulated rats (Table 3), but embryo recovery on day 5 was still significantly lower than oocyte recovery on day 1. This partial recovery of embryos in the a/s-treated rats probably results from the reduction in postovulatory oestradiol concentrations (Figure 9), although other factors may be involved. A similar reduction in postovulatory oestradiol concentrations has been observed in superovulated cattle following treatment with PMSG a/s (Kummer et al., 1980). Use of inhibitors of oestradiol synthesis, to reduce postovulatory oestradiol concentrations, were only partially successful in increasing embryo recovery rates. AGP, a general inhibitor of steroid synthesis, and 4AA, a more specific inhibitor, resulted in similar recovery rates to those seen after PMSG a/s (Figure 29). However, an increase in the proportion of abnormal embryos in the absence of significantly decreased ovarian oestradiol

concentrations, was observed after 4AA treatment (Table 17).

The increased preovulatory oestradiol concentrations in PMSG a/s-treated rats, compared to FSH infused animals, had detrimental effects on implantation 5-6 days later. Blastocysts from these a/s-treated rats were normal (Table 18), but the ability of the uteri of these animals to undergo decidualization was significantly impaired (Table 19). Earlier work (Armstrong and Greep, 1965; Koch and Oettel, 1977) had also shown that superovulation with PMSG could adversely affect decidualization. This effect of preovulatory oestradiol on implantation is similar to the results observed by Butcher et al (1969a and b) who used delayed ovulation to prolong exposure of the intrafollicular oocyte to oestrogen.

The results presented in this thesis can therefore be summarized as follows:

1. A superovulatory dose of PMSG (40 i.u.) can induce the ovulation of a large number of follicles in immature rats. However, after mating almost all the embryos are lost before day 5 of pregnancy. This infertility occurs as a result of problems in several of the stages from ovulation to implantation.
2. Not all oocytes ovulated by superovulated rats are capable of undergoing fertilization at the time when spermatozoa are present in the reproductive tract, if animals are caged with male rats on the evening of day 0. This is due to the long time span over which ovulation occurs in these rats. Ovulation occurs from 22 h after PMSG onwards, with a burst of ovulations from 2400 h on day 0 to 0600 h on day 1, at a time comparable to that seen in control rats.

3. Although rats were only mated overnight of day 0 to day 1, oocytes collected at earlier times are capable of undergoing fertilization and normal development if transferred to a mated recipient close to the anticipated time of ovulation of these oocytes.
4. In vivo fertilization of oocytes in cumulus, collected from superovulated rats, is lower than that of control rats, suggesting either a reduction in the fertilization rate, or a less discrete timing of ovulation overnight of day 0 to day 1 in superovulated rats.
5. The developmental capacity of oocytes collected from the oviducts of superovulated donors, early on the morning of day 1, is equal to that of control rats, when assessed by transfer to a synchronized mated recipient.
6. Loss of a large number of embryos occurs over days 1-3 of pregnancy in superovulated rats and almost all embryos are lost by day 5. These losses can be partially overcome by administration of a PMSG antiserum at 1800 h on day 0.
7. The loss of viable embryos cannot be overcome by placing ligatures at the uterotubal junction, or at both the uterotubal junction and close to the ampulla. Embryo recovery rates are increased by placing ligatures but the proportion of degenerate embryos recovered also increases.
8. Concentrations of serum and ovarian oestradiol are significantly elevated in superovulated rats after ovulation. It is suggested that the oestradiol may be detrimental to embryo survival, but

the possibility of other compounds being detrimental cannot be excluded. Reduction of postovulatory oestradiol concentrations to those seen in control animals, by use of a general inhibitor of steroid synthesis (AGP) results in an embryo recovery rate similar to that observed after use of a PMSG antiserum. Use of a more specific aromatase inhibitor (4AA) does not reduce oestradiol concentrations as much, but embryo recovery rates are comparable to treatment with AGP. However, use of 4AA results in an increased proportion of abnormal and degenerate embryos.

9. If a PMSG antiserum is used to ensure the presence of blastocysts in the uterus on day 5, implantation only occurs in ~ 50% of rats, which suggests abnormalities in the blastocysts, uterine environment or both. The blastocysts are normal, as shown by transfer to the uterus of a synchronized recipient. In contrast, the ability of the uterus to undergo decidualization is impaired in many rats, suggesting that the pregnancy failure can be attributed to abnormalities in the uterus.
10. A further small loss of pregnancy occurs in superovulated rats treated with antiserum, after implantation, with the result that only ~ 29% of animals are pregnant by day 20. Most of these losses occur soon after implantation.
11. Superovulation with a continuous infusion of FSH produces a comparable number of oocytes to 40 i.u. PMSG but subsequent embryo losses are considerably lower. All rats show implantation sites on day 8 and pregnancy continues to day 20 in 80% of rats. Ovarian oestradiol content are never as high as in 40 i.u. PMSG -

treated rats, which again suggests that the losses in the latter are attributable to the elevated oestradiol content.

The use of the immature rat as a model for the infertility following excessive gonadotrophic stimulation has enabled the following general conclusions to be drawn:

1. Superovulation with exogenous gonadotrophins is a useful technique for producing large numbers of viable oocytes.
2. The superovulatory treatment may indirectly decrease fertility or cause complete infertility as a result of the production of an abnormal hormonal environment.
3. In particular, excessive oestrogen production by the hyperstimulated ovary may subsequently have adverse effects on the maternal reproductive tract.

Caution must always be exercised in extrapolating from one species to another. However, if the immature rat can be used as a model for the events occurring after the induction of ovulation in both humans and domestic livestock, then the following considerations are applicable. There is no evidence to suggest that superovulation with exogenous gonadotrophins results in the production of abnormal oocytes, and the use of gonadotrophic preparations in both humans and domestic livestock may be a valuable technique for increasing the yield of oocytes. In domestic livestock the increased numbers of oocytes allows for the multiplication of genetically superior stock and subsequent progeny testing. In humans, mild superovulation, to avoid the complication of ovarian hyperstimulation syndrome, increases



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the probability of collecting mature oocytes, capable of undergoing in vitro fertilization.

Following superovulation, however, the maternal environment may no longer be optimum for embryonic development or implantation. In domestic livestock, where superovulation can be combined with embryo transfer, this problem can be overcome by transfer of the embryos to a synchronized recipient which has not been subjected to excessive gonadotrophic stimulation. However, in humans, where the objective is to transfer the embryos back to the donor, problems with the maternal environment may be encountered. In this case a compromise must be reached between stimulation to produce a reasonable number of oocytes, and overstimulation of the maternal environment, which may have adverse effects on embryo survival. Alternatively, techniques developed for use in animals and recently applied to human research may allow for the stimulation of the ovaries during one cycle, collection and in vitro fertilization of the oocytes, followed by freezing of the embryo and transfer of the embryo back to the donor during a subsequent unstimulated cycle. This technique may also prove valuable in women, by allowing for the collection of sufficient oocytes so that embryos can be transferred to the uterus at interval during the woman's life. Thus, more than one pregnancy can be achieved from a single superovulatory treatment and the trauma of multiple superovulations and laparoscopies can be avoided.

If the adverse effects of the maternal environment are attributable to excessive production of oestrogen or some other steroid by the hyperstimulated ovary, then it may be possible to

neutralize or inhibit production of the harmful factor. However, pharmacological compounds, or even antiserum, must be used with extreme caution during pregnancy because of the possibility of causing abortion or teratogenesis. In addition, long term effects on the foetus must be considered, particularly when human reproduction is involved.

Despite the problems demonstrated, the potential exists for the production of large numbers of viable oocytes by the use of exogenous gonadotrophins, if the abnormalities subsequent to ovulation can be overcome.

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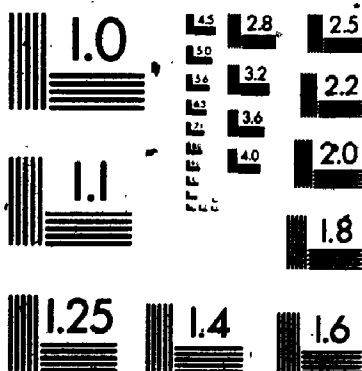
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